Oncogenic properties of a spermatogenic meiotic variant of Fer kinase expressed in somatic cells


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Running head: Tumor formation promoting activity of FerT

Conflict of interest: Etai Yaffe, Yoav Elkis, Sally Shpungin and Uri Nir, are shareholders of the "Urifer" Biotech, start-up company

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Abstract

The kinase Fer and its spermatogenic meiotic variant, FerT, are co-expressed in normal testes and cancerous tumors, but whether they exert related roles in spermatogenic or malignant cells has not been known. Here we show that Fer and FerT reside in the mitochondria of spermatogenic cells and are harnessed to the reprogrammed mitochondria of colon carcinoma (CC) cells. Both kinases bound complex I of the mitochondrial electron transport chain (ETC) in spermatogenic and CC cells and silencing of either Fer or FerT was sufficient to impair the activity of this complex. Directed mitochondrial accumulation of FerT in non-malignant NIH3T3 cells increased their ETC complex I activity, ATP production and survival, contingent upon stress conditions caused by nutrient and oxygen deprivation. Strikingly, directed mitochondrial accumulation of FerT endowed non-malignant cells with tumor-forming ability. Thus, recruitment of a meiotic mitochondrial component to cancer cell mitochondria highlights a pivotal role for reprogrammed mitochondria in tumorigenesis.

Keywords: Fer, FerT, mitochondria, electron transport chain (ETC), cancer, tumor.
Introduction

The study of mitochondrial bioenergetics has been dedicated to understanding the central role of the mitochondrion in cellular ATP generation. Generation of ATP occurs via the respiratory electron transport chain (ETC), which is coupled to the mitochondrial oxidative phosphorylation (Oxphosph.) processes (1). However, in addition to its essential role in cell energy and metabolism, the mitochondrion is now recognized as a multifunctional organelle, which participates in a variety of cellular processes including proliferation, death and senescence (2,3). Deregulation of mitochondrial functions, in particular at the level of the ETC, is associated with several pathologies, including cancer (4). Cancer cells adopt mitochondrial alterations and metabolic re-programming in order to sustain their unique metabolic needs and produce the molecules and energy required to promote tumor growth (5). Specifically, while normal mammalian cells primarily utilize mitochondrial Oxphosph. for ATP production, cancer cells remodel their glycolytic and mitochondrial machinery so that glycolysis is up-regulated but only marginally replenishes the mitochondrial tricarboxylic acid cycle (TCA cycle) and the Oxphosph. processes, even under aerobic conditions (6). This phenomenon of prevailing aerobic glycolysis is termed the "Warburg effect" (7-10). It should be noted, that in parallel to the documented relevance of aerobic glycolysis to cancer cell metabolism, recent studies have also established the importance of mitochondrial processes in malignant transformation (11).

Fer is an intracellular tyrosine kinase, which resides in both the cytoplasm and nucleus of mammalian cells (12). Together with c-Fes, Fer constitutes the F-BAR domain-containing subfamily of intracellular tyrosine kinases that share a unique structure with an extended N-terminal tail [Previously reviewed in (13,14)]. Fer is involved in the regulation of several cellular processes. These include among others, "outside-in" signaling which regulates cytoskeleton organization and cell adhesion (13,15-17). Fer has also been implicated in the response of cells to stress cues. It was shown to rescue cells from ionic radiation (18), and to mediate the migration of fibroblasts in response to reactive oxygen species (19). Several lines of evidence also suggest a supportive role of Fer in the progression and growth of malignant tumors. The kinase was detected in all human malignant cell lines analyzed (20,21) and its levels in malignant prostate tumors are significantly higher than those detected in benign growths (22). Furthermore, down-regulation of Fer impairs the proliferation of prostate, breast and colon carcinoma (CC) cells (23), induces death in CC and non-
small cell lung cancer (NSCLC) cells (24,25), abolishes the ability of prostate carcinoma PC3 and V-Sis-transformed cells to form colonies in soft agar (22) and prevents the metastatic spread of breast and lung adenocarcinoma tumors (26,27). At the clinical level, high Fer expression levels have been linked to poor prognosis of hepatocellular-carcinoma (HCC) (28), clear cell renal cell carcinoma (29,30), postoperative NSCLC (25), and high-grade basal/triple-negative breast cancer (26).

A truncated variant of Fer, termed FerT, is encoded by a testis-specific fer transcript. FerT shares common SH2 and kinase domains (KD) with Fer, but the two enzymes differ in their N-terminal tails. Whereas the N-terminal tail of Fer encompasses 412 aa, the N-terminal portion of FerT is 43 aa long and it is devoid of all the functional N-terminal elements found in the Fer enzyme (21,31).

FerT was reported to accumulate in primary spermatocytes residing in the first meiotic prophase (32,33) as well as in post-meiotic spermatids (34). While absent from normal somatic tissues, FerT was found to accumulate in CC and HCC cells, and its down regulation impairs the proliferation and survival of these malignant cells (24). However, the molecular mechanisms underlying the role of this meiotic protein in cancer cell proliferation and survival has not been revealed.

In the current study, we show that FerT and Fer populate the re-programmed mitochondria of colon carcinoma (CC) cells, and that directed accumulation of FerT in mitochondria of non-cancerous cells suffices to endow them with the ability to form tumors in-vivo.

Materials and Methods

Antibodies and Reagents

The following antibodies were used for either immuno-blotting or immunocytochemistry: Affinity purified anti-N-terminal Fer (anti-N-Fer) antibodies (24), affinity purified anti-SH2 Fer/FerT antibodies (anti-Fer/FerT) (35), anti-complex I NDUFAB subunit monoclonal antibody (MitoSciences), anti-VDAC, anti-mitochondria monoclonal antibody (Meridian), anti-porin 31HL (Ab-2) monoclonal antibody (Calbiochem), anti-ATP5A mitochondrial ATP synthase alpha subunit monoclonal antibody (Santa Cruz Biotechnology), anti-mitochondrial Complex IV (Cox IV) (3E11, Cell Signaling, USA), anti-c-Myc (9E10, sc-40, Santa Cruz biotechnology, USA), anti-Regulator of chromosome condensation 1 (RCC1) (sc-374325, Santa Cruz Biotechnology, USA),...
anti-caspase 3 (Santa Cruz Biotechnology, sc-56053), anti-actin antibodies (I-19) (Santa Cruz Biotechnology), anti-alpha tubulin (Santa Cruz Biotechnology), and anti-lamin B monoclonal antibody (Santa Cruz Biotechnology).

**Sperm cell isolation, capacitation and protein lysate preparation**

Ejaculated bull semen was obtained from the Israeli Veterinary Institute (Bet-Degan, Israel), and washed three times by centrifugation (780 × g, 10 min) in NKM buffer (110 mM NaCl, 5 mM KCl, and 10 mM N-morpholino propanesulfonic acid pH 7.4). *In vitro* capacitation and protein lysate preparation of bull sperm were carried out as described before (36).

Mouse mature caudal spermatozoa were obtained by dissecting the cauda epididymis of male ICR mice followed by its puncture for 15 minutes in a dish containing 3 ml PBS to enable sperm release out of the epididymal tissue. The sperm suspension in PBS was then filtered through a nylon mesh with 50 μm pores, and centrifuged at 1000 × g for 5 minutes; the pellet was re-suspended either in a lysis buffer for protein analysis or in fresh PBS solution.

**Immunocytochemistry**

Cells were spread, fixed and stained with the appropriate primary antibody, as described (37). The immuno-stained slides were inspected under an AxioimagerZ1 (Zeiss, Germany) fluorescence microscope or a confocal fluorescence microscope (Olympus-FV1000).

**Cell culture, normoxia, nutrient starvation, and hypoxia growth conditioning**

Cell lines (HCT116, NIH3T3, SW620) were obtained from the American Type Culture Collection (ATCC). Cell authentication was carried out using a morphology check by microscope and by characterization of the DNA profiles using short tandem repeat analysis. HCT116 and SW620 cells were grown in Minimum Essential Medium (MEM) containing: 10% heat-inactivated fetal bovine serum (FBS) and 1% nonessential amino acids (Biological Industries, Beth Haemek, Israel). NIH3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). All cells were grown at 37°C with 5% CO₂.

For nutrient starvation, DMEM medium was aspirated 24h after cell seeding and replaced by Hank's balanced salt solution (Biological Industries, Israel) for 24h.
For growth under hypoxia, plates with NIH3T3 cells grown in DMEM were transferred for 24 h to anaerobic culture jar containing carbon dioxide generating envelope (GasPak™ EZ, BD, USA) that reduces the oxygen level in the jar to 1% within 30 min.

**Isolation of mitochondria and preparation of cytoplasmic and mitochondrial extracts**

Intact mitochondria were isolated from hearts, livers and testes of ICR mice and from HCT116 colon carcinoma cells. Tissues were excised from 10 week old wild type mice and collected into 10 volumes of ice-cold isolation buffer (IB) and mitochondria were isolated as described (38).

**Immuno-precipitation**

Purified mitochondria were lysed and complex I or V were immune-captured from 1 mg protein using specific beads according to the manufacturer (Mito Sciences) instructions. Total mitochondrial lysates or immuno-precipitates were resolved by SDS-PAGE and immuno-blotted with specific antibodies.

**siRNA mediated silencing of the fer and ferT genes**

siRNA mediated silencing in cells was carried out as described (39). siRNAs sequences are presented in Table S1.

**ROS measurements**

ROS levels were measured as described (39).

**Measurement of the mitochondrial OXPHOS complex I activity**

Activity of the mitochondrial OXPHOS Complex I (NADH dehydrogenase) was determined using the Microplate Assay for Human Complex I Activity (MS141-MitoSciences), according to the manufacturer’s instructions.

**Plasmid construction and DNA transfection of cells**

A Myc-tag encoding sequence was ligated to the 5′ of the human ferT cDNA (24) that was already cloned in the p-EFIRES-P (pIREs) expression vector (40), constructing the p-EFIRES-MycFerT (MycFerT), plasmid. The mitochondrial targeting sequence (MTS) derived from the integral mitochondrial protein cytochrome c oxidase subunit VIII (11) was than ligated to the 5′ of
the Myc- human FerT cDNA in the above described vector to form the p-EFIRES-MTS-MycFerT (MTS-MycFerT) plasmid. A kinase dead FerT (MTS-MycFerT<sup>G201R</sup>) (41) was constructed by introducing a point mutation to the p-EFIRES-MTS-MycFerT plasmid, using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, USA) with the following primers: 5'-cttcaccaaaatttcctttgcgcagtaattctcccaatatgac-3', 5'-gtcatattgggagaat tactgcgcaaaggaaattttggtgaag-3' according the manufacture instructions. The MycFerT, MTS-MycFerT and MTS-MycFerT<sup>G201R</sup> encoding plasmids were used to stably transfect NIH3T3 cells, using the transfection reagent Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions.

**Quantification of cellular ATP and NAD levels**

Cells were suspended in 400 µl cold 0.4M perchloric acid solution in 1.5 ml tubes. The mixture was incubated on ice for 15 minutes and was then centrifuged at 12,000 ×g to remove the precipitate. The supernatant was neutralized with 4M K<sub>2</sub>CO<sub>3</sub> solution to pH 7.5, followed by incubation for 15 minutes on ice. Following centrifugation (10,000 ×g), the supernatants were immediately used for chromatographic analysis. The chromatography apparatus consisted of Hitachi Elite LaChrom system equipped with diode array detector, column oven, autosampler, and a quaternary pump. All chromatographic analyses were performed at 30°C using SUPELCOSIL™ LC-18-S HPLC Column (5 µm particle size, L × I.D. 25 cm × 2.1 mm), flow rate 0.2 mL/min under isocratic elution conditions with the following buffer composition: [50 mM potassium phosphate, 100 mM triethylamine, pH 6.5 (adjusted with phosphoric acid)];Acetonitrile (98.5:1.5)]. Each analysis cycle was set to 30 minutes. The chromatographic flow was monitored at 260 nm and integrated using EZChrom Elite Software.

**Cell survival assays**

Death levels in transfected NIH3T3 cells were determined by using the MultiTox-Fluor Multiplex Cytotoxicity Assay (G9201, Promega, USA) according to the manufacturer’s protocol or by Annexin V and propidium iodide (PI) fluorescent staining of cells, as described (24,39).
Tumor formation assay in mice

All animal experiments were performed according to the guidelines of the Bar-Ilan Institutional Animal Care and Use Committee. Mice were housed five per cage with unlimited access to food and water, and exposure to 12h light/12h dark cycles.

Ten, 8 weeks old female immuno-compromised nude mice (Harlan, Israel) were subcutaneously injected with $4 \times 10^6$ NIH3T3 transfected cells and tumor volume was determined as described before (37).

Immunohistochemical (IHC) analysis of frozen tumor sections

Tumors from nude mice were dissected and fixed as previously described. Sections were stained with the appropriate antibody by a standard IHC staining protocol (37) using anti-c-Myc (9E10, sc-40, Santa Cruz Biotechnology, USA) followed by incubation with a secondary Alexa Fluor® 488 goat anti–mouse IgG antibody (Molecular Probes, Invitrogen, Paisley, U.K.). Nuclei were visualized by staining with 0.1 $\mu$g/ml Hoechst.

The immuno-stained sections were viewed with an Axioimager z1 microscope.

Statistical analysis

Statistical analysis was performed using the paired and unpaired Student's $t$-test, with a $P$ value <0.05 considered significant.

Results

Fer and FerT reside in the mitochondria of spermatogenic and malignant cells

We previously showed that down-regulation of Fer increases ROS level in CC cells (39). To examine whether FerT similarly inhibits the generation of ROS in malignant cells, the protein was knocked-down in CC cells which endogenously express both Fer and FerT, using different, specific siRNA sequences (Fig. 1A and Table S1). This led to a significant increase in the cellular level of ROS, to an extent that was similar to the one measured in Fer depleted cells (Fig. 1B and Fig.S1 A and B). The stimulatory effect of Fer and FerT depletion on the elevation of ROS production, suggested the regulatory involvement of these proteins in cellular ROS generating processes. These can take place either outside or within the mitochondria (42). Additional support for the presence of Fer or FerT in the mitochondria is provided by the association of Fer with Stat3 (31) and the recent
detection of Stat3 in the mitochondria of mammalian cells (11). We therefore examined the accumulation of Fer and FerT in the mitochondrion of normal cells.

Since both Fer and FerT accumulate in spermatogenic cells (32,43), we first determined whether the two proteins are co-expressed in mature spermatozoa, as they are in malignant cells. We initiated our study with mature spermatozoa, which can be reliably isolated and identified. Both enzymes were detected in whole cell lysates prepared from mouse caudal epididymal spermatozoa and from bovine ejaculated sperm (Fig. 2A).

To determine the sub-cellular localization of Fer in sperm cells, ejaculated bovine sperm cells were immuno-cytochemically stained with affinity purified anti-N-Fer antibodies. The observed immuno-staining was confined to the sperm mid-piece section, which primarily represents the mitochondrial sheath of these cells (44). Furthermore, the staining of Fer overlapped the immuno-staining of the mitochondrial ATP synthase 5a subunit (ATP5A) when examined by fluorescence microscopy (Fig. 2B). To further substantiate the overlapping signals of the Fer and ATP synthase 5a subunit immuno-staining, confocal microscopy analysis was applied. This approach showed clear co-localization of Fer and the mitochondria (Fig. 2C), thereby indicating the presence of Fer in the mitochondria of sperm cells.

To further corroborate this finding, and to examine whether also FerT resides in the mitochondria of spermatogenic cells, we performed a sub-cellular fractionation analysis. Proteins were extracted from cytoplasmic and mitochondrial fractions prepared from mouse testicular cells. Fer and FerT were found to reside in the mitochondrial fraction, whose purity was confirmed using specific mitochondrial markers: Voltage-dependent anion channel (VDAC) protein (associated with the outer mitochondrial membrane), and NDUFA9 [NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9-associated with complex I of the ETC] proteins. α-tubulin, which normally resides in the cytoplasm and the regulator of chromosome condensation 1 (RCC1), whose accumulation is confined to the cell nucleus, served as extra-mitochondrial markers (Fig. 3A). Similarly, Fer was present in mitochondrial fractions prepared from mouse liver and heart tissues, which prominently express this tyrosine kinase (Fig. 3B and C).

Fer and FerT are both expressed in CC cells (24). We therefore examined whether these two proteins also reside in the mitochondria of malignant cells. Similarly to testicular cells, both Fer and FerT were detected in mitochondria purified from the HCT116 CC cells (Fig. 3D).
To more directly verify the presence of Fer in the mitochondria of malignant cells, affinity purified anti-N-Fer antibodies were applied in an immuno-cytochemical analysis. Staining of HCT116 CC cells with anti-N-Fer antibodies and with a specific anti-mitochondrial monoclonal antibody, revealed overlapping staining of the two antibodies, demonstrating the presence of Fer in the mitochondria of malignant cells (Fig. 3E). Collectively, these observations substantiate the accumulation of Fer and FerT in the mitochondria of CC cells.

**Fer and FerT associate with complex I of the ETC in testicular and CC cells and support complex I activity**

The mitochondrial presence of Fer or FerT, and the increased ROS production upon down-regulation of either of these proteins, suggested their association with the respiratory ETC components in malignant cells. Since generation of mitochondrial ROS is linked to ETC complex I activity (45), we examined the association of Fer and FerT with this complex and with complex V, which functions down-stream in the ETC. Complexes I and V were immuno-precipitated from normal and from malignant cells, using a monoclonal antibody that captures components of these complexes. To ensure specificity of the immuno-precipitation, precipitated proteins were challenged with antibodies directed towards mitochondrial proteins which are part of either complex I or complex V, or which associate with the mitochondrial membrane. These included the complex I component, NDUFA9, ATP5A (ATP synthase complex 5a subunit), which is part of the ETC complex V, and VDAC. As expected, NDUFA9 co-immuno-precipitated with the anti-complex I antibody from mitochondrial extracts of testicular, heart and liver cells. ATP5A, which served as negative controls, was not precipitated by this antibody (Fig. 4A), indicating the specificity of the assay. Interestingly, while Fer and FerT co-precipitated with complex I from the mitochondria of testicular cells (Fig. 4A), Fer did not associate with complex I precipitated from mitochondria of normal heart or liver (Fig. 4B).

To examine the association of Fer and FerT with mitochondrial complex I of malignant cells, the complex was immuno-precipitated from SW620 and HCT116 CC cells. Both Fer and FerT, as well as Stat3, co-precipitated with complex I prepared from mitochondria of the two malignant cell types, while VDAC which served as a negative control in these experiments was not precipitated (Fig. 4C and D). However, the three proteins did not co-precipitate with complex V prepared from...
SW620 cells (Fig. 4C). Thus, similarly to testicular cells, Fer and FerT associate with complex I, in the mitochondria of malignant CC cells.

Based on the above results, we hypothesized that Fer or FerT take part in the mitochondrial functioning in CC cells. To test this notion, we examined the effect of Fer or FerT depletion on the functioning of the ETC - complex I. Fer or FerT were knocked-down using specific siRNAs, and the activity of the complex was measured in solubilized mitochondria. Down-regulation of either Fer or FerT in CC cells decreased complex I activity by approximately 40% (Fig. 4E).

**Directed mitochondrial accumulation of FerT salvages complex I activity, ATP production and cell survival under nutrient and oxygen-deprivation conditions**

To further explore the outcome of mitochondrial accumulation of Fer and FerT in somatic cells, we directed ectopic accumulation of the two enzymes in mitochondria of NIH3T3 cells. Myc-tagged Fer and FerT were each fused in the N-terminus of the protein to a mitochondrial targeting sequence (MTS) derived from the cytochrome c oxidase subunit VIII, an integral mitochondrial protein (11). NIH3T3 cells were stably transfected with the Fer and FerT expressing vectors (40), or with the empty vector alone (NIH3T3-pIRES cells). The mitochondrial accumulation of the two ectopic proteins was examined using immuno-fluorescence. While Fer could not be stably and ectopically overexpressed in the mitochondria of NIH3T3 cells (not shown), the expression of MTS- MycFerT was stable and was confined to the mitochondria of the transfected cells (NIH3T3-MTS-MycFerT cells) (Fig. 5A).

The directed mitochondrial accumulation of FerT might endow the transfected cells with an increased efficiency of complex I activity and ATP production. We therefore measured the mitochondrial complex I activity and the steady state levels of ATP in NIH-pIRES and NIH3T3-MTS-MycFerT cells grown under normal and stress conditions. The most significant difference between complex I activity in mitochondria of NIH3T3-MTS-MycFerT cells in comparison to NIH-pIRES cells, was observed when starvation conditions were applied. Notably, this significant effect of FerT on complex I was observed for kinase active FerT but not for kinase-inactive FerT mutant (MTS-MycFerT<sup>G201R</sup>) (41) (Fig. 5B). The enhanced activity of complex I was further corroborated by an observed increase in the level of the complex I primary metabolite NAD (45), in starved NIH3T3-MTS-MycFerT cells (Fig. 5C). Notably, the difference in complex I activity under
starvation coincided with the relative levels of ATP which were measured in the two cell types grown under this stress condition. A profound increase of 37% in the cellular ATP level was seen in NIH3T3-MTS-MycFerT cells compared to the NIH3T3- pIRES cells, when the two cell types were grown under nutrient and growth factor deprivation conditions (Fig. 5D). To examine whether NIH3T3- pIRES and NIH3T3-MTS-MycFerT cells undergo apoptotic death under stress conditions, the presence of apoptotic markers was analyzed in lysates prepared from the two cell types. The apoptotic marker cleaved caspase 3 was detected only in hypoxic NIH3T3-pIRES cells but not in NIH3T3-MTS-MycFerT. Nor was this marker detected in NIH3T3-pIRES or NIH3T3-MTS-MycFerT cells grown under nutrient starvation condition (Fig. 6A). The increased apoptotic death in hypoxic NIH3T3-pIRES cells was further corroborated by an increased staining of these cells by annexin V and propidium iodide (PI), under hypoxia (Fig. 6B). A higher percentage of dead NIH3T3-pIRES cells in comparison to NIH3T3-MTS-MycFerT cells grown under nutrient starvation, was seen when the Multiplex-cytotoxicity assay has been applied (Fig. 6C). Thus, directed expression of FerT increases the survival of NIH3T3 cells under stress growth conditions.

**Directed mitochondrial accumulation of FerT is required and sufficient to drive tumor formation in-vivo**

To directly examine the role of mitochondrial FerT in tumor initiation and/or progression, we examined the ability of NIH3T3 cells expressing FerT either bearing (NIH3T3- MTS-MycFerT, cells) or lacking MTS (NIH3T3-MycFerT cells) to drive the formation of tumors in-vivo. NIH3T3 are immortal cells in culture, and we therefore assumed that the ultimate criteria for deciphering an effect of FerT on their malignant potency, is to examine their ability to form tumors in mice. Control NIH3T3-pIRES cells, NIH3T3- MTS-MycFerT or NIH3T3-MycFerT cells were subcutaneously injected to immuno-compromised (nude) mice. Strikingly, in all 10 injected animals, 28 days post-injection, MTS-MycFerT expressing cells started to develop highly vascularized tumors, which after 42 days reached an average size of 1 cm³. Notably, the NIH3T3-MycFerT cells, expressing FerT which lacks MTS (Fig. S2A) developed small tumors in only 3 out of 10 injected mice, by day 42. No tumor developed during this period of time by NIH3T3-pIRES cells harboring the empty expression vector, pIRES (Fig. 7A, B, Fig. S2B and Table S2). Immuno-blotting of tumor protein lysates and immuno-staining of tumor sections confirmed the ectopic presence of
MTS-MycFerT in the elicited tumors (Fig. 7C and D). Thus, guided mitochondrial accumulation of FerT is required and sufficient to endow non-malignant cells with tumor-forming capacity.

Discussion

Reprogrammed metabolism and altered energy generation pathways are hallmarks of the abnormal survival and proliferation of malignant cells (7-10). Although functionally modified, cancer cell mitochondria are not globally dysfunctional, but are rather reprogrammed to support the unique metabolic needs of malignant cells (46,47). Moreover, mitochondrial ATP production replenishes aerobic glycolysis in cancer cells, by its coupling to the functioning of the key glycolytic enzyme, Hexokinase II, which is physically associated with the mitochondrial outer membrane anchored protein, VDAC1 (48). Hence, cancer cell mitochondria may adopt unique tools to direct and support their modified functioning. In the current study, we show that Fer and its spermatogenic, meiosis specific variant, FerT, can provide such a function. We found that Fer and FerT associate with complex I of the mitochondrion ETC in spermatogenic and in CC cells. Accordingly, down-regulation of either one of these proteins impaired the functionality of this complex and increased cellular ROS levels. Thus, Fer and FerT are part of the metabolic reprogramming system which restrains the accumulation of ROS and their potential damaging effects (49) in CC cells. In spermatogenic cells, the association of Fer and FerT with complex I, and the maintenance of low ROS levels should reduce the risk of DNA damage caused to chromosomal DNA undergoing extensive recombination in primary spermatocytes that harbor relatively high levels of Fer and FerT (50). In sperm, Fer and FerT may enhance the production of ATP which is propelling the motility of this cell and aid the sperm cells avoiding energy depletion and death due to low availability of nutrients and oxygen.

Computational analysis failed to identify MTS in either Fer or FerT. Thus, the translocation of these enzymes to the mitochondria of spermatogenic or CC cells may depend on their co-transport with a protein which is present in these cells and does bear MTS. Similar possibility was raised for the Stat3 protein which lacks MTS and trans-locates to the mitochondria (11).

Our findings portray Fer and FerT as mitochondrial proteins that can support energy generation and restrict ROS production in sperm and in malignant cells. The harnessing of Fer and FerT to the reprogrammed mitochondria of malignant cells enables these cells to improve their energy
production under restricted availability of essential nutrients like glucose. This enables cancer cells to cope with stress conditions that are prevalent in solid tumors when malignant cells outgrow the carrying capacity of the local vasculature. Several reported findings envisage a proliferation-supportive role of Fer, in malignant cells grown under stress conditions. For example, the requirement of Fer for the de-regulated proliferation of cancer cells in-vitro is most profoundly manifested when the cells are grown in agarose under non-anchoring conditions accompanied by restricted nutrient and oxygen availability (51). Furthermore, shRNA knock-down studies carried out with mouse xenografts models showed that Fer plays an important role during the initiation of primary tumors (51), and is essential for the development of secondary metastases (26,27). This might reflect, at least in part, a required contribution of Fer to the initiation and early stages of tumor formation, during which the nutrient and oxygen supplying vasculature has not yet been established. However, the molecular mechanisms through which Fer exerts this supportive activity in tumor formation remained elusive. Based on our current study we propose that Fer and FerT support tumor initiation and formation through their mitochondrial functions. Similar role was reported for the Fer interacting mitochondrial protein Stat3 (31), which is required for the proliferation of RAS-transformed cells grown in agarose, under non-adherent and nutrient restrictive conditions (11). In accordance with this notion, we found in the current study that mitochondrial FerT augments the activity of the ETC complex I in transfected cells and that this is accompanied by a significant increase in the cellular ATP pool, thereby enabling cellular survival, under nutrient and oxygen deprivation conditions. Thus, FerT enables CC cells to survive nutrient and oxygen deprivation conditions, which are experienced by these cancerous cells during tumor formation. This is most probably carried out by a FerT directed up-regulation of the initiation rate of the mitochondrial oxidative phosphorylation process as is reflected by an increase in NAD and ATP levels, under these stress conditions. Notably, while the increased survival of FerT expressing cells under nutrient deprivation could be linked to an increased activity of complex I, the increased survival of NIH3T3-MTS-MycFerT cells under hypoxia seems to be complex I independent and may reflect an anti-apoptotic activity of FerT in the mitochondria. The effects of FerT on the mitochondrial complex I activity, could be either kinase dependent or independent. Using a kinase inactive FerT we found that the effect of FerT on complex I is at least in part kinase activity dependent.
The significance of the mitochondrial role of FerT in tumor formation is most profoundly demonstrated by the ability of MTS-MycFerT to endow NIH3T3 cells with the capacity to efficiently form fast growing tumors in all mice injected with these FerT expressing cells. Unlike this observation, MTS lacking FerT drove the formation of slowly growing tumors in only 30% of the injected animals. It should also be noted that FerT was previously shown to exert only minor tyrosine kinase activity in the cytoplasm of transfected somatic cells, and that this activity is significantly lower than that exerted by Fer (21,31). We therefore assume that the major effect of FerT on tumor formation by the transfected cells results predominantly from its mitochondrial activity, rather than from a putative de-regulated tyrosine kinase activity in the cytoplasm.

Collectively, our data suggest that modified mitochondrial functioning can endow immortal cells with the ability to form tumors. Thus, reprogrammed mitochondria can drive the initiation and progression of tumors. Identification of key components in the mitochondrial reprogramming process should therefore offer new targets for cancer intervention.

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Figure legends

**Figure 1.** Down-regulation of Fer or FerT induces ROS levels in CC cells. A. Protein level of Fer and FerT in HCT116 CC cells 48h after transfection with control or specific siRNA. Fer and FerT were detected using anti-Fer/FerT antibodies by western-blot analysis. B. ROS levels were measured 48h after transfection of cells with the different siRNAs. Histograms represent means +/- SE (n=4).

**Figure 2.** Expression of Fer and FerT in spermatozoa. A. Sperm cell lysates were prepared from ejaculated bovine sperm incubated in capacitation medium or left un-capacitated, and from mouse caudal epididymis spermatozoa. Proteins were resolved by 10% SDS-PAGE, and Fer and FerT were detected using anti-Fer/FerT antibodies in a western-blot analysis. AKAP3 was used as a sperm-specific marker. B. Immunocytochemical staining of bovine ejaculated sperm for Fer (anti-N- Fer antibodies- red) and ATP synthase 5 α subunit (anti-ATP5A- green). The diffraction interference contrast (DIC) and the merged images are shown in the bottom of the figure. Bars represent 10 µm. These images represent one out of three independent experiments, which gave similar results. C. Confocal microscopic images of immunocytochemical stained bovine ejaculated sperm for Fer (red) and ATP5A (green). Bars represent 10 µm. Merged images are shown in the lower panels. Images boxed in red and white are enlarged in the right upper and lower images respectively. These images were obtained with the Zeiss LSM 510 confocal microscope with consecutive sections of 0.7 µm, and they represent one out of four independent experiments, which gave similar results.

**Figure 3.** Fer and FerT reside in the mitochondria of testicular and malignant cells and Fer accumulates in the mitochondria of normal somatic tissues. Tissues homogenates from: A. mouse testes, B. livers and C. hearts, and lysates from HCT116 cells D. were fractionated into whole cell extract (WCE), whole cell after nuclear clearance (CE2 - nuclear), cytoplasmic after mitochondria removal (-mito), and mitochondrial fractions. Protein extracts from all fractions were resolved by SDS-PAGE. Fer and FerT were detected using anti-Fer/FerT antibodies, NDUFA9 and VDAC served as mitochondria-specific markers, α tubulin as a cytoplasmic marker, and RCC1 as a nuclear marker. All proteins were detected using specific antibodies by western-blot analysis. These images
shown represent one out of three independent experiments, which gave similar results.

**E.** HCT116 cells were fixed and stained with Hoechst 33342 for nuclear visualization (blue) (a), with anti-mitochondria antibody (green) (b), and with anti-N-Fer antibodies (red) (c); merged images (d). Boxed area in (d) is enlarged in (e), arrow indicates co-localization. Dashed box area in (d) is enlarged in (f). Images were obtained with the Zeiss LSM 510 confocal microscope with consecutive sections of 0.7 μm.

**Figure 4.** Fer and FerT associate with the mitochondrial ETC complex I and support its activity. **A.** Protein extracts were prepared from cytoplasmic (cyto) and mitochondrial (mito) fractions of mouse testicular cells. ETC Complex I was immuno-precipitated from the mitochondrial extract (IP:mitoCxI). Proteins from all samples were resolved by SDS-PAGE. Fer and FerT were detected with anti-Fer/FerT antibodies by western-blot analysis. NDUFA5 (complex I) and ATP5A (complex V) served as markers for immuno-precipitation selectivity. **B.** Protein lysates were prepared from cytoplasmic and mitochondrial fractions of mouse liver and hearts. Complex I was immuno-precipitated from the mitochondrial fractions, and all marked proteins were detected using specific antibodies in a western-blot analysis. **C.** Protein lysates were prepared from cytoplasmic and mitochondrial fractions of SW620 CC cells. Complex I (upper panels) and complex V (lower panels) were immuno-precipitated from the mitochondrial fractions. Precipitation with IgG conjugated beads (IP: IgG beads, upper panels) served as a control for the specific immuno-precipitation of complex I (upper panels). IP-Supp. in the lower panels denotes the supernatant of the immuno-precipitation with anti-complex V antibody. All presented proteins were detected using specific antibodies in a western-blot analysis. **D.** Complex I was immuno-precipitated from mitochondrial fraction of HCT116 CC cells. All proteins were resolved by SDS-PAGE and the presented proteins were detected using specific antibodies by western-blot analysis. All presented images represent one out of three independent experiments, which gave similar results. **E.** Knock-down of Fer or FerT impairs the ETC complex I activity. HCT116 cells were treated with either control, scrambled (siRNA-scr), siRNA directed towards fer mRNA (siRNA-fer), or siRNA directed towards ferT mRNA (siRNA-ferT). Complex I was isolated from each sample, and its activity was measured...
spectrophotometrically, as described in the Methods. The activities in Fer and FerT depleted cells were normalized to the control (siRNA-scr) activity, which was given a value of 100. Histograms represent means +/- SE (n=3).

**Figure 5.** Mitochondrial presence of FerT salvages complex I activity and ATP accumulation under stress conditions. A. NIH3T3 cells were transfected with either the empty pIRES vector (NIH3T3-pIRES) (a-d) or with a plasmid encoding MTS-MycFerT (NIH3T3-MTS-MycFerT) (e-h). Cells were fixed and stained with Hoechst 33342 to visualize nuclei (blue), with anti- complex IV for mitochondria (red), and with anti-Myc for FerT (green). (i) Enlarged image depicting the accumulation of MTS-MycFerT in a typical mitochondrial distribution profile in transfected NIH3T3 cells (NIH3T3-MTS-MycFerT). B. Complex I activity in NIH3T3-pIRES (pIRES) and NIH3T3-MTS-MycFerT (FerT) cells grown under normal and hypoxic conditions. Under starvation conditions the activity in these cells was compared to cells expressing FerT kinase-domain inactive mutant (FerTKDmut). Values +/- SE (n=3). C. Cellular NAD levels in NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells grown under starvation conditions. Histograms represent average values +/- SE (n=5). D. Cellular ATP levels in NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells grown under normal and stress conditions. Histograms represent average values +/- SE (n=5).

**Figure 6.** Reduced death of NIH3T3-MTS-MycFerT cells grown under stress conditions. A. NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells were grown under nutrient starvation or hypoxia for 24 h. Protein lysates were prepared, resolved by SDS-PAGE, and reacted with anti-caspase 3 and anti-actin antibodies by western blot. The gel shown represents one out of three independent experiments, which gave similar results. B. Annexin V and PI staining of NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells grown under hypoxic conditions. Histograms represent average values +/- SE (n=3). C. Viability of NIH3T3-pIRES and NIH3T3-MTS-MycFerT cells under starvation. The percentage of dead cells was determined for each cell type using the MultiTox-Fluor Multiplex Cytotoxicity Assay. Histograms represent average values +/- SE (n=7).

**Figure 7.** Directed mitochondrial accumulation of FerT drives the formation of tumors in-vivo. A. Representative pictures of NIH3T3-MTS-MycFerT tumors (black arrows)
and NIH3T3-pIRES areas of injection (white arrows) in immuno-compromised nude mice. B. Tumors were measured 6 weeks post inoculation, and the average size in each group is presented +/- SE (n=10). C. Tumors were removed and protein lysates were prepared, separated by SDS-PAGE and subjected to anti-Myc antibodies by western-blot analysis to detect the MTS-MycFerT protein. Results of two representative tumors are presented. D. Frozen sections were prepared from the NIH3T3-MTS-MycFerT tumors, and were stained with anti-Myc antibodies (green) to detect the MTS-MycFerT protein. Nuclei were visualized with Hoechst 33342 (blue). Sections of two representative tumors are presented.
Figure 1

A.

<table>
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<tr>
<th>siRNA:</th>
<th>scramble</th>
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<tr>
<td>Actin</td>
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1  2  3

B.

Fold increase of ROS level

- **siRNA-neg**
- **siRNA-fer**
- **siRNA-ferT**

* P<0.05  n=4
Figure 2

A. Bovine ejaculated Sperm

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Mouse Sperm

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B. DIC

Fer

ATP5A

Merge

C. Merged

Fer

ATP5A

Merge

Merge 10µm
Figure 6

(A) Western blot analysis of Caspase-3, Cleaved Caspase-3, and Actin under starvation and hypoxia conditions with pIRES and FerT conditions.

(B) Bar graph showing the percentage of stained cells under annexin, annexin+PI, and PI conditions with pIRES and FerT conditions.

(C) Bar graph showing the percentage of dead cells under pIRES and FerT conditions.

* P<0.05  n=3

* P<0.05  n=7
Figure 7

A. Mouse 1, Mouse 2, Mouse 3, Mouse 4

B. 

![Bar chart showing average tumor volume](chart.png)

- *P < 0.001  n=10
- **P < 0.05  n=10

C. Tumor 1, Tumor 2

D. Tumor 1, Tumor 2

IB: c-Myc
Oncogenic properties of a spermatogenic meiotic variant of Fer kinase expressed in somatic cells

Etai Yaffe, Elad Hikri, Yoav Elkis, et al.

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