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***In vitro* effects of *FBXW7* mutation in serous endometrial cancer: increased levels of potentially druggable proteins and sensitivity to SI-2 and dinaciclib**

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Abstract

Serous endometrial cancers (ECs) are clinically aggressive tumors that frequently harbor somatic mutations in *FBXW7* (*F-box and WD repeat domain-containing 7*). The *FBXW7* tumor suppressor is part of a SCF (complex of SKP1, Cullin 1, F-box protein) ubiquitin ligase complex which controls the degradation of numerous substrates that, if not properly regulated, can contribute to the initiation or progression of tumorigenesis. Despite reports that up to 30% of serous ECs include somatic mutations in *FBXW7*, the molecular effects of mutated *FBXW7* in ECs have not been determined. Here, we used transient transfection and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) editing in serous EC cell lines to interrogate the molecular effects of six recurrent *FBXW7* mutations. We show that *FBXW7* mutations lead to increased Cyclin E1, steroid receptor coactivator 3 (SRC-3), c-MYC, Rictor, glycogen synthase kinase 3 (GSK3), P70S6 kinase, and protein kinase B (AKT) phosphorylated protein levels in serous EC cells. Furthermore, we demonstrate that CRISPR-edited *FBXW7*-mutant ARK1 serous EC cells exhibit increased sensitivity to SI-2 (a SRC inhibitor) and dinaciclib (a cyclin dependent kinase (CDK) inhibitor) compared to parental ARK1 cells. Collectively, our findings reveal biochemical effects of *FBXW7* mutations in the context of EC and provide *in vitro* evidence of sensitivity to targeted inhibitors.

Keywords

uterine cancer; ARK1; ARK2; Cyclin E1; SRC-3

Introduction

It is estimated that more than 300,000 new cases and 76,000 deaths due to endometrial cancer (EC) occur worldwide each year ¹. Women with the most common histological subtype, endometrioid EC, can frequently be cured through hysterectomy. However, women with rare and more clinically aggressive forms of EC are more often diagnosed once the disease has spread beyond the uterus, have a high propensity to relapse, and commonly face a poorer prognosis ^{2–5}. These rare subtypes include serous and clear cell ECs, as well as

uterine carcinosarcomas. Five-year disease-free survival rates for women with serous or clear cell EC are 18–55%^{5–8} and 38–50%^{8–10}, respectively; five-year overall survival rates for women with uterine carcinosarcoma are only 6–38%^{11–13}.

FBXW7 (*F-box and WD repeat domain containing 7*; also known as *FBW7* and *CDC4*) somatic mutations are among the most common genomic aberrations in serous ECs, clear cell ECs, and carcinosarcomas. *FBXW7* is a tumor suppressor that serves as the substrate recognition subunit of a SCF (complex of SKP1, Cullin 1, F-box protein) ubiquitin ligase complex and has been implicated in the regulation of at least 35 substrates across cancer types¹⁴, many of which are oncoproteins. The *FBXW7* gene encodes three primary isoforms (α , β , and γ) that differ only at the N-termini¹⁵. *FBXW7* α , the most abundant isoform, targets the most substrates that have been tested experimentally, and localizes to the nucleoplasm; the β and γ isoforms localize to the cytoplasm and nucleolus, respectively^{16, 17}. In all histological subtypes of EC, *FBXW7* mutations occur predominantly as missense mutations, including hotspot mutations at codons 423, 465, 479, and 505 in the substrate-recognition domain (WD repeats) and at codons 658 and 689 carboxy terminal to the WD repeats. We and others have reported somatic *FBXW7* mutations in 17–30% of serous ECs^{18–21}, 11–28% of uterine carcinosarcomas^{22–26}, 7–25% of clear cell ECs^{8, 24}, and 3–10% of endometrioid ECs^{8, 18, 21, 27}.

Despite the high frequency of occurrence, little is known of the molecular consequences of *FBXW7* mutations in ECs. Thus far only indirect correlations between increased Cyclin E protein and *FBXW7* mutations have been shown^{15, 19}, leading to speculation that *FBXW7* mutations might dysregulate Cyclin E in EC. In keeping with this idea, one study reported that *FBXW7* mutations and genomic deletions occur mutually exclusively of *CCNE1* amplification in serous ECs¹⁹, although others found no association across histological subtypes of EC²⁸. Herein, we provide novel insights into the functional consequences of *FBXW7* mutations in serous EC cells. We show that recurrent somatic *FBXW7* mutations cause increased levels of phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT proteins in serous EC cell lines. Furthermore, we provide *in vitro* evidence that CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-edited *FBXW7*-mutant ARK1 serous EC cells exhibit increased sensitivity to SI-2 (SRC3 inhibitor-2) and dinaciclib (a cyclin dependent kinase (CDK) inhibitor) compared to parental ARK1 cells.

Materials and Methods

Cell culture

ARK1 and ARK2 serous EC cell lines were established²⁹ and kindly provided by Dr. Alessandro A. Santin (Yale University School of Medicine, New Haven, CT). Both lines are wildtype with respect to *FBXW7* mutation status; ARK1 exhibits *FBXW7* copy number loss²⁰. Cell lines were maintained in Roswell Park Memorial Institute medium (RPMI) + 10% FBS at 37°C in a humidified atmosphere with 5% CO₂. Short tandem repeat (STR) profiling by American Type Culture Collection (ATCC) in 2014 verified that both cell lines were human and did not match any profile in the ATCC or German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany) databases. Aliquots of cells frozen in 2014 and stored in liquid nitrogen were used for shRNA and transient transfection experiments. After

return from Washington University's Genome Engineering and iPSC Center (GEIC, St. Louis, MO), parental ARK1 cells were re-profiled in 2017 by Laragen Inc. (Culver City, CA) and authenticated to the 2014 profile; results verified that ARK1 did not match any other cell line profile in the ATCC or DSMZ databases. STR profiles of the ARK1 CRISPR-edited cell lines authenticated to parental cells. For all experiments, cell numbers were determined using a Countess Cell Counter (Thermo Fisher Scientific, Waltham, MA).

Short hairpin RNA (shRNA) infection

ARK1 cells were plated in 96 well plates (1.6×10^4 cells/well) and incubated 24hrs before media was replaced with media containing 8 μ g/ml polybrene (EMD Millipore, Burlington, MA). MISSION[®] lentiviral transduction particles containing shRNA directed against *FBXW7* (TCRB0000368359, Sigma-Aldrich, St. Louis, MO) or Non-Mammalian shRNA Control Transduction Particles (SHC002V) were added at multiplicity of infection (MOI) of 2. Cells were incubated for 18hrs before media was replaced. After 24hrs, cells were replated and maintained in selection media (0.5 μ g/mL puromycin (Sigma-Aldrich, St. Louis, MO) in RPMI + 10% FBS) until lysate collection 2–3 weeks later. Infections were repeated in 4 biological replicates.

FBXW7 α construct subcloning / DNA isolation

Dr. Philip Hieter (The University of British Columbia, Vancouver, BC) kindly provided the *FBXW7 α* wildtype construct to which we added SrfI and SalI restriction sites before subcloning the *FBXW7 α* insert into the pCMV-3Tag-7 vector (Agilent Technologies, Inc., Santa Clara, CA). We mutagenized the pCMV-3Tag-7-*FBXW7 α* wildtype construct using the QuikChange II Site-directed Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's instructions. Mutagenized inserts were excised and subcloned again into the pCMV-3Tag-7 vector. DNA was extracted from bacterial cultures using the QIAGEN Plasmid Plus Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The following coding (and encoded protein) changes, reported with respect to NCBI accession number NM_033632 based on the hg18 assembly of the human genome sequence, were incorporated and verified using Sanger sequencing: c.G1268T (p.G423V), c.C1393T (p.R465C), c.G1394A (p.R465H), c.G1436A (p.R479Q), c.C1513T (p.R505C), and c.C2065T (p.R689W).

Transient transfections

Cells were plated in 60mm dishes (3×10^5 cells/dish (ARK1) or 3.15×10^5 cells/dish (ARK2)) and incubated at 37°C in 5% CO₂ for 24hrs prior to transfection of 2.5 μ g of *FBXW7 α* constructs (or vector control) with X-tremeGENE[™] HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Lysates were collected 24hrs after transfection as described below for a minimum of 3 biological replicates.

Protein isolation / quantification

For transient transfections, cells were plated and collected as described above. CRISPR cell lines were collected 24hrs after plating (3×10^5 cells/60mm dish). Cells were scraped into

PBS and lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) containing cOmplete™ mini protease inhibitor tablets (Roche Applied Science, Mannheim, Germany), 0.1mM NaVO₄ and 1mM NaF (New England Biolabs, Ipswich, NJ). Protein concentration was determined using the QuickStart Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). For dinaciclib/DMSO-treated lysates, cells were plated in 60mm dishes (3×10⁵ cells/dish) and incubated at 37°C in 5% CO₂ for 24hrs prior to addition of media containing 100nM dinaciclib or equivalent volume of DMSO. Media was collected 24hrs after treatment, and adherent cells were scraped into 1mL remaining media. Floating and scraped cells were combined, washed twice in PBS and lysed in RIPA buffer as described above.

Western blotting

Equal amounts of protein were separated on 8% or 10% Bis-Tris Plus Gels (Thermo Fisher Scientific, Waltham, MA). Protein was wet-transferred in Tris-glycine transfer buffer containing 20% methanol to PDVF membranes (Bio-Rad Laboratories, Hercules, CA). Blots were blocked in tris-buffered saline containing 1% Tween 20 (TBST) + 5% milk. We used the following antibodies from Cell Signaling Technology (Danvers, MA) according to their protocols: AKT (#4685), Phospho (P)-AKT (Ser473) (#4060), Aurora A (#91590), BCL-2 (#15071), BCL-XL (#2764), cleaved caspase 3 (#9664), cleaved caspase 7 (#8438), cleaved caspase 9 (#7237), cleaved PARP (#5625), c-MYC (#5605), P-c-MYC (Ser62) (#13748), Cyclin E1 (#4129), P-Cyclin E1 (Thr62) (#4136), GSK3α/β (#5676), P- GSK3α/β (Ser21/9) (#8566), MCL-1 (#4572), MYC-tag (#2272), mTOR (#2972), p53 (#2527), P70S6 (#2708), P-P70S6 (Thr389) (#9205), P-Rb (#8516), Rictor (#2114), P-Rictor (Thr1135) (#3806), SRC-3 (#2126), P-SRC-3 (Thr24) (#2979), survivin (#2802), anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (#7074) and anti-mouse IgG HRP-conjugated secondary antibody (#7076). Blots were incubated in FBXW7 antibody (A301–720A, Bethyl Laboratories, Inc., Montgomery, TX) overnight at 4°C at a dilution of 1:1000 in TBST + 5% milk after multiple freeze/thaws, following a previously published protocol³⁰. We verified specificity of the antibody for FBXW7α using immunoprecipitation with Surebeads™ Protein A Magnetic Beads (Bio-Rad Laboratories, Hercules, CA) and comparison to protein size of the *FBXW7α* wildtype construct. We incubated blots in β-Actin antibody (A2228; Sigma-Aldrich) overnight at 4°C at a dilution of 1:500 in TBST + 5% milk. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) or Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA) prior to film exposure (Carestream Health Inc., Rochester, NY or Agfa Healthcare NV, Belgium) and development using a film processor (Konica Minolta, Ramsey, NJ). To enable probing of blots for multiple antibodies, blots were incubated in Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA). All Western blots were replicated in a minimum of 3 biological replicates.

CRISPR-editing

ARK1 cells were CRISPR-edited by Washington University's Genome Engineering and iPSC Center (GEIC, St. Louis, MO). Single guide RNAs (sgRNAs) were assembled into a plasmid (43860; Addgene, Cambridge, MA), validated using deep sequencing³¹, and evaluated using Washington University's GEIC's proprietary off-target profile program (Supplemental Table 1). One million ARK1 cells were transiently transfected with 0.2–0.5μg

green fluorescent protein (GFP), 1.5µg sgRNA, 2.5µg Cas9 (43945; Addgene, Cambridge, MA), and 100pmol of single-stranded oligodeoxynucleotide (ssODN) donor templates (Integrated DNA Technologies, Skokie, Illinois) (Supplemental Table 2) using a 4D-Nucleofector™, X unit (Lonza, Basel, Switzerland). GFP+ cells were sorted four days post-nucleofection. The following genomic edits, reported with respect to NCBI accession number NM_033632 based on the hg18 assembly of the human genome sequence, were incorporated and verified using Sanger sequencing: c.G1268T (p.G423V), c.C1393T (p.R465C), c.G1394A (p.R465H), c.G1436A (p.R479Q), c.C1513T (p.R505C), and c.C2065T (p.R689W) (Supplemental Figure 3).

DNA isolation / polymerase chain reaction (PCR) amplification/Sanger sequencing

DNA was isolated from cell lines using the Gentra® Puregene® Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. *FBXW7* primer sequences were previously reported ²¹; PCR conditions are available upon request. PCR reactions were subjected to exonuclease I (Epicentre, Madison, WI)/shrimp alkaline phosphatase (Affymetrix, Santa Clara, CA) purification prior to Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA). Sequence traces were reviewed using Sequencher™ 5.4.6 (Gene Codes Corporation, Ann Arbor, MI).

Cell viability and apoptosis assays

Cells were plated in 96-well plates (5000 cells/well; 3 wells/dose) and treated with inhibitors or equivalent concentrations of DMSO (Sigma-Aldrich, St. Louis, MO) 24hrs later. Cell viability and caspase 3/7 activity were determined using the Promega CellTiter-Glo® Viability Assay and Caspase-Glo® 3/7 Assay, respectively, according to the manufacturer's protocol (Promega, Madison, WI) in a minimum of 3 biological replicates. Dinaciclib (SCH727965), seliciclib (Roscovitine, CYC202), and SI-2 were purchased from Cayman Chemical Company (Ann Arbor, MI). Vistusertib (AZD2014), MK2206 2HCl, everolimus (RAD001) and sapanisertib (INK128, MLN0128) were acquired from Selleck Chemicals (Houston, TX).

Flow Cytometry

Cells were plated in 6-well plates (2.3×10^5 cells/well) and incubated 24hrs before replacing media with media containing dinaciclib or equivalent volume of DMSO (Sigma-Aldrich, St. Louis, MO). After 24hrs of treatment, cells were analyzed using the NUCYCL™ PI kit (Exalpha Biologicals Inc., Shirley, MA) or the Calbiochem® Annexin V-FITC Apoptosis Detection Kit (Millipore Sigma, Burlington, MA) according to the manufacturer's protocols. Unstained cells were used as negative controls and experiments were repeated in 3 biological replicates. Data were analyzed using FlowJo software v10 (FlowJo LLC, Ashland, OR).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Results

Phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT protein levels increase in serous EC cells transiently expressing mutant FBXW7 α compared to those expressing wildtype FBXW7 α

To initially select proteins to be included in analysis of molecular effects of *FBXW7* mutations, we searched publications for potential substrates of FBXW7 and studied the aberration pattern of the encoding genes in The Cancer Genome Atlas's (TCGA's) data of serous-like ECs. We prioritized ten genes that were frequently (10%–27%) amplified and for which a majority of amplifications occurred independent of *FBXW7* mutations: *CCNE1*, *NCOA3*, *MYC*, *RICTOR*, *NOTCH3*, *TP63*, *MCL1*, *KLF2*, *MAP7D1*, and *HSF1* (Supplemental Figure 1a). Using Western blotting, we assessed the effect of *FBXW7* shRNA depletion on proteins encoded by eight out of ten of these genes in ARK1 serous EC cells; we were unable to detect TP63 and KLF2 using commercially available antibodies. Of these eight proteins, NOTCH3, MCL-1, MAP7D1 and HSF1 did not reproducibly exhibit increased levels following *FBXW7* shRNA knockdown (data not shown). In contrast, *FBXW7* shRNA knockdown in ARK1 serous EC cells resulted in increased phosphorylated Cyclin E1, SRC-3, c-MYC, and Rictor protein levels (Supplemental Figure 1b). These results suggest that Cyclin E1, SRC-3, c-MYC, and Rictor proteins are endogenously regulated by FBXW7 in ARK1 serous EC cells.

To begin to assess whether *FBXW7* mutations affect Cyclin E1, SRC-3, c-MYC, and Rictor protein levels in the context of EC, we generated MYC-tagged expression constructs for six *FBXW7 α* mutations which encode the following protein changes: FBXW7 α ^{G423V}, R465C, R465H, R479Q, R505C, and R689W. Each of these mutations is recurrent in EC; furthermore, of *FBXW7*-mutated serous ECs, 57% harbor one of these somatic mutations^{18, 19, 21}. All but FBXW7 α ^{R689W} occur within the WD repeat domain, which is responsible for substrate binding^{14, 32} (Supplemental Figure 2).

We transiently transfected mutant and wildtype *FBXW7 α* constructs in ARK1 and ARK2 serous EC cells and assessed protein expression using Western blotting. We found increased Cyclin E1, SRC-3, and c-MYC in cells transiently expressing *FBXW7 α* mutants compared to those expressing *FBXW7 α* wildtype, with some exceptions with respect to FBXW7 α ^{R689W} (Figure 1a, b). Specifically, phosphorylated SRC-3 and c-MYC were not increased in cells transiently expressing FBXW7 α ^{R689W} compared to cells expressing wildtype FBXW7 α .

Phosphorylated Rictor protein levels were also increased in ARK1 and ARK2 cells transiently expressing *FBXW7 α* mutations, except FBXW7 α ^{G423V}, compared to those expressing wildtype FBXW7 α (Figure 1c, d). In light of these observations and given that SRC-3 and Rictor can activate AKT^{33–35} and that AKT activation can result in SRC-3 activation via inhibitory phosphorylation of GSK3³⁶, we determined the effects of *FBXW7 α* mutations on components of the AKT pathway. We found that phosphorylated GSK3, P70S6, and AKT proteins were increased in cells expressing *FBXW7 α* mutations, except FBXW7 α ^{G423V}, compared to those expressing wildtype FBXW7 α (Figure 1c, d). The highest levels of phosphorylated GSK3, P70S6, and AKT proteins were observed in

cells expressing the more C-terminal FBXW7 α mutations, a pattern that was also observed for total SRC-3 and phosphorylated Rictor (Figure 1). Collectively, our results indicate that transient exogenous expression of recurrent FBXW7 α mutations in serous EC cells results in dysregulation of one or more proteins evaluated and highlight differences in the functional consequences of WD-domain mutants and the more distal FBXW7 α ^{R689W}.

CRISPR-edited *FBXW7*-mutated ARK1 serous EC cells exhibit increased phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT protein levels compared to parental cells

Transient transfections may have limitations, including variability in expression across constructs and replicates, and, with respect to *FBXW7*, isoform-specific expression. Therefore, we CRISPR-edited ARK1 cells to insert the same *FBXW7* mutations we investigated using transient transfections. Sanger sequencing of all *FBXW7* exons verified that only the desired edit was incorporated into each cell line except FBXW7^{R465H}; these cells harbored the intended c.G1394A (p.R465H) and an unintended mutation, c.T1391_G1392insT(p.R465Afs*7) (Supplemental Figure 3). This cell line is therefore referred to hereafter as FBXW7^{R465H/R465Afs*7}. In addition to the desired genomic edit, wildtype *FBXW7* was detected in all CRISPR-edited lines except FBXW7^{G423V} and FBXW7^{R465H/R465Afs*7} (Supplemental Figure 3).

Consistent with results obtained using transient transfection, we found increased levels of phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT proteins in CRISPR-edited *FBXW7*-mutant ARK1 cells compared to parental cells, with two exceptions: phosphorylated c-MYC was not increased in FBXW7^{R465C} or FBXW7^{R689W} cells (Figure 2). Also similar to our transient transfection observations, protein levels of Cyclin E1 were increased in CRISPR-edited FBXW7^{R689W} ARK1 cells compared to parental, but to a lesser extent than other *FBXW7*-mutant cells.

Differences between results obtained using 24hr transient transfections (Figure 1) and CRISPR-edited cell lines (Figure 2) may be expected, considering differences in exposure time to *FBXW7* mutations and that only the alpha isoform of *FBXW7* was transiently transfected, although results from either experimental setup may reflect biologically important effects. We found three differences in comparing the results of these experimental approaches. First, as mentioned above, phosphorylated c-MYC was not increased in CRISPR-edited FBXW7^{R465C} cells compared to parental ARK1 cells, which differs from the increase observed in cells transiently expressing this mutation compared to cells expressing wildtype FBXW7 α . Second, unlike in cells transiently expressing FBXW7 α ^{R689W}, CRISPR-edited FBXW7^{R689W} cells exhibited increased levels of phosphorylated SRC-3 compared to parental cells. Third, CRISPR-edited FBXW7^{G423V} cells exhibited increased phosphorylated Rictor, GSK3, P70S6, and AKT compared to parental cells, results inconsistently observed in cells transiently expressing FBXW7 α ^{G423V} compared to cells expressing wildtype FBXW7 α . This difference may reflect the lack of detectable wildtype *FBXW7* in CRISPR-edited FBXW7 α ^{G423V} cells. Apart from these three differences, there was overall agreement between results of transient transfections and

CRISPR-edited ARK1 cells; recurrent *FBXW7* mutations result in increased phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT protein levels.

Compared to parental cells, CRISPR-edited *FBXW7*-mutant ARK1 cells exhibit decreased viability and increased apoptosis in response to SI-2 and dinaciclib

We hypothesized that the increased protein levels detected in association with *FBXW7* mutations might promote sensitivity to targeted inhibitors and used cell viability assays to test this possibility. To indirectly target increased Rictor and Cyclin E1 protein levels, we treated cells with three mTOR inhibitors (everolimus, vistusertib, and sapanisertib) and two CDK inhibitors (seliciclib and dinaciclib), respectively. To directly target increased SRC-3 and AKT, we used SI-2 and MK2206, respectively. The viability of CRISPR-edited *FBXW7*-mutant ARK1 cells did not differ from that of parental cells in response to the three mTOR inhibitors tested, MK2206, or seliciclib (Supplemental Figure 4), with the exception of *FBXW7*^{R465C} cells which showed a reduction in cell viability in response to everolimus (Supplemental Figure 4c). In contrast, we found statistically significantly decreased viability in CRISPR-edited *FBXW7*-mutant ARK1 cells compared to parental cells in response to SI-2 and dinaciclib.

SI-2 is a small molecule SRC inhibitor that selectively targets SRC-3 over SRC-1 or -2³⁷. After treatment with a range of SI-2 concentrations selected based on *in vitro* data³⁷, CRISPR-edited *FBXW7*-mutant ARK1 cells exhibited significantly decreased viability compared to parental cells (Figure 3a). The decrease in viability was accompanied by a significant increase in apoptosis, measured by caspase 3/7 activity, in *FBXW7* mutant cells compared to parental ARK1 cells (Figure 3b).

Dinaciclib inhibits CDK2 (which is activated by Cyclin E1) as well as CDK5, 1 and 9³⁸. CRISPR-edited *FBXW7*-mutant cells exhibited significantly decreased viability after treatment with dinaciclib (at doses equivalent to plasma concentrations achieved in humans³⁹) compared to parental ARK1 cells (Figure 4). Treatment with dinaciclib induced significantly more apoptosis, measured by caspase 3/7 activity and/or annexin V staining, in CRISPR-edited *FBXW7*-mutant ARK1 cells compared to parental cells, with exceptions of *FBXW7*^{R465H/R465Afs*7} and *FBXW7*^{R689W} (Figure 5a, b; Supplemental Figure 5). In support of increased apoptotic induction, we detected higher levels of cleaved PARP, cleaved caspase 3, cleaved caspase 7, and/or cleaved caspase 9 in CRISPR-edited *FBXW7*-mutant ARK1 cells compared to parental cells (Figure 5c). Decreases in MCL-1, BCL-XL and Survivin in cells treated with dinaciclib compared to cells treated with dimethyl sulfoxide (DMSO) were further evidence of apoptotic induction (Figure 5c). Interestingly, in the absence of dinaciclib, CRISPR-edited *FBXW7*-mutant ARK1 cells exhibited increased MCL-1 and Survivin protein levels, but decreased BCL-2, compared to parental cells (Figure 5c).

In addition to increased apoptosis, different changes in cell cycle distributions were induced by dinaciclib treatment in CRISPR-edited *FBXW7*-mutant ARK1 cells than were induced in parental cells (Figure 6a, Supplemental Figure 6). After treatment with 10nM dinaciclib, ARK1 parental cells exhibited no change in cell cycle distribution compared to DMSO control (Figure 6a). In contrast, four out of six ARK1 mutant lines exhibited significant

changes after 10nM dinaciclib treatment compared to DMSO controls: FBXW7^{R465H/R465Afs*7} and ^{R479Q} exhibited decreased percentages of cells in G1, FBXW7^{R505C} exhibited an increased percentage of cells in S phase, and FBXW7^{R689W} exhibited an increased percentage of cells in G2/M phase. At higher concentration of dinaciclib treatment (100nM), the percentage of ARK1 parental cells (and FBXW7^{R689W} cells) in G1 phase significantly increased while the percentages in S and G2/M decreased compared to DMSO controls, indicating G1 cell cycle arrest or delayed progression through G1 (Figure 6a). In contrast, FBXW7^{G423V} and FBXW7^{R465C} cells exhibited signs of delayed progression through G2/M phase after 100nM dinaciclib treatment: decreased percentages of FBXW7^{G423V} and FBXW7^{R465C} cells in S phase and a significant increase in the percentage of FBXW7^{G423V} cells in G2 phase were observed (Figure 6a). FBXW7^{R465H/R465Afs*7}, ^{R479Q}, and ^{R505C} cells exhibited less of a G1 arrest in response to 100nM dinaciclib compared to that observed in parental cells: no significant decreases in the percentages of FBXW7^{R465H/R465Afs*7}, ^{R479Q}, and ^{R505C} cells in S phase were observed, and the percentage of FBXW7^{R505C} cells in G2/M did not significantly decrease in response to dinaciclib treatment (Figure 6a). As expected, dinaciclib treatment decreased levels of phosphorylated Rb, as well as other cell cycle proteins, compared to levels in DMSO-treated cells (Figure 6b). Interestingly, in the absence of dinaciclib, p53 protein levels were increased in *FBXW7*-mutant ARK1 cells compared to parental cells (Figure 6b). In summary, these findings indicate that CRISPR-edited *FBXW7*-mutant ARK1 cells exhibit increased sensitivity to SI-2 and dinaciclib compared to parental ARK1 cells; molecular underpinnings of this sensitivity include induction of apoptosis and/or changes in cell cycle distribution.

Discussion

Despite the high prevalence of somatic *FBXW7* mutations in ECs, particularly in clinically aggressive subtypes such as serous and clear cell ECs, as well as uterine carcinosarcomas, the molecular consequences of these mutations in the context of EC remain unknown. Here, we used transient transfection and CRISPR-editing to interrogate the molecular effects of *FBXW7* mutations in serous EC cell lines. We show that *FBXW7* mutations result in increased phosphorylated SRC-3, Cyclin E1, c-MYC, Rictor, GSK3, AKT and P70S6 protein levels. Furthermore, we provide *in vitro* evidence of increased sensitivity of *FBXW7*-mutant serous EC cells to SI-2, a SRC inhibitor, and dinaciclib, a CDK inhibitor.

SRC-3, also known as AIB1 and NCOA3, is a *FBXW7* substrate³⁶ and oncoprotein that plays a role in integrating signals from a number of pathways important in tumorigenesis⁴⁰. Phosphorylation of SRC-3 at Thr24, an event that we show is increased in association with *FBXW7* mutation, is important for the interaction of SRC-3 with NF-KB, SRC-3 activity associated with IL-6 gene regulation and the transforming ability of SRC-3⁴¹. SRC-3 mRNA has been shown to be upregulated in EC compared to normal endometrium⁴² and EC patients with higher SRC-3 mRNA expression have poorer prognoses compared to patients with relatively lower expression⁴³. To our knowledge, the present study is the first to show an association between *FBXW7* mutations and increased phosphorylated SRC-3 protein levels in EC cells. Furthermore, we show that *FBXW7*-mutated CRISPR-edited

ARK1 serous EC cells are more sensitive than parental cells to SI-2, a small molecule inhibitor of SRC that selectively targets SRC-3 over SRC-1 or SRC-2³⁷.

Like SRC-3, Cyclin E1 is oncogenic upon dysregulation⁴⁴ and is a known FBXW7 substrate^{32, 45}, but experimental evidence of direct association between *FBXW7* mutation and increased Cyclin E1 protein levels in EC has not to our knowledge been described prior to the results provided herein. A previous study provided an indirect correlation by showing increased Cyclin E1 protein by immunohistochemistry in serous ECs with *FBXW7*^{R465C} or *FBXW7*^{R465H} mutation¹⁹. Others have shown increased phosphorylated Cyclin E by Western blot analysis in ECs with point mutations in the WD repeats (*FBXW7*^{R465H}, *R479Q*, and four nonsense mutations in the WD repeats), but not in tumors with mutations in the amino terminus of *FBXW7*¹⁵.

Our analysis of one recurrent carboxy-terminal mutation (*FBXW7*^{R689W}) supports this idea that mutations outside the WD repeats may not produce equivalent biochemical effects as those within. *FBXW7*^{R689W} cells exhibited lower levels of phosphorylated SRC-3 and Cyclin E1 compared to cells harboring a mutation within the WD repeats and were less sensitive to SI-2 and dinaciclib compared to most other cells with a WD repeat domain mutation. In contrast to the lower levels of phosphorylated SRC-3 and Cyclin E1 in *FBXW7*^{R689W} cells compared to those with WD repeat domain mutations, *FBXW7*^{R689W} cells exhibited some of the highest levels of phosphorylated Rictor, AKT, GSK, and P70S6 proteins.

Increased phosphorylated Rictor levels in *FBXW7*^{R689W} cells, combined with the observation that transient transfection of wildtype *FBXW7* α did not diminish Rictor protein levels, leads us to hypothesize that Rictor is not a direct substrate of *FBXW7* in serous EC cells. However, the purpose of this study was not to define substrates of *FBXW7*, but rather to identify molecular effects of *FBXW7* mutation in the context of EC. In this regard, increased phosphorylated Rictor, AKT, GSK, and P70S6 protein levels in cells with some *FBXW7* mutations might reflect altered cellular signaling related to dysregulation of *FBXW7* substrates. In support of this idea, Rictor associates with *FBXW7* in the regulation of c-MYC and Cyclin E⁴⁶; Rictor phosphorylation (Thr1135) by AKT or P70S6^{46, 47} promotes dissociation of Rictor from Cullin-1⁴⁸ and promotes AKT phosphorylation (Ser473)^{33, 34}; phosphorylated AKT can be induced by SRC-3 overexpression³⁵ and, inversely, can promote phosphorylated SRC-3 (Thr24) through GSK inhibitory phosphorylation (Ser9/21)³⁶. Future work is needed to determine if increases in Rictor, AKT, GSK3 and P70S6 protein levels associated with *FBXW7* mutations reflect druggable changes.

A caveat of this study is that our search for molecular effects of *FBXW7* mutations was biased by an initial focus on proteins that were previously published as *FBXW7* substrates in any cellular context, that were frequently aberrant independent of *FBXW7* mutation in TCGA's serous-like tumors, and for which levels increased following *FBXW7* shRNA knockdown in serous EC cells. Rictor, c-MYC, SRC-3 and Cyclin E1 met these criteria; we subsequently investigated AKT, GSK, and P70S6 because of shared pathway interactions with Rictor and SRC-3^{34–36, 47, 48}. This approach was not designed to interrogate all

possible molecular consequences of *FBXW7* mutation and may also have failed to identify some molecular effects involving proteins considered in the initial shRNA experiments. For example, we did not focus on MCL-1 because protein levels only increased in two out of four replicates of *FBXW7* shRNA knockdown in ARK1 cells. However, subsequent Western blotting for MCL-1 as a pro-survival marker revealed consistently increased protein levels in DMSO-treated *FBXW7*-mutant ARK1 cells compared to parental cells (Figure 5c). These results indicate that increased MCL-1 protein levels are a molecular effect of *FBXW7* mutation, but perhaps not of complete *FBXW7* depletion, in ARK1 serous EC cells. Similarly, the increased p53 protein levels in DMSO-treated *FBXW7*-mutant cells compared to parental cells suggests increased p53 may be a molecular effect of *FBXW7* mutation in serous EC cells (Figure 6b).

Our combined results provide an impetus for future experiments. For example, although our comparison of the drug sensitivity of *FBXW7*-mutant CRISPR-edited ARK1 cells to parental cells was ideal in terms of correlating response to *FBXW7* mutation status, subsequent work is needed to determine if *FBXW7* mutations in EC cells with different genetic backgrounds result in increased sensitivity to SI-2 and dinaciclib. Furthermore, large-scale drug screens using, and proteomic analysis of, CRISPR-edited *FBXW7*-mutant EC cells may reveal additional druggable proteins and other molecular effects of *FBXW7* mutations. The implications of this study will also need to be tested in organoids or *in vivo* models.

In conclusion, the results presented herein provide new insights into the molecular effects of *FBXW7* mutations in serous ECs. We identified increased levels of a number of phosphorylated proteins associated with recurrent *FBXW7* mutations, most of which occur within druggable pathways. Furthermore, we provide *in vitro* evidence that *FBXW7* mutations confer sensitivity to SI-2 and dinaciclib. Combined, these novel biochemical results lay the groundwork for future studies to determine whether *FBXW7* mutations represent an Achilles' heel that could be leveraged to improve the care of women suffering from clinically aggressive ECs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ATCC	American Type Culture Collection
AKT	protein kinase B

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CDK	cyclin dependent kinase
DMSO	dimethyl sulfoxide
EC	endometrial cancer
FBXW7	F-box and WD repeat domain-containing 7
G1	Gap 1 phase of cell cycle
G2/M	Gap 2/mitosis phase of cell cycle
GSK3	glycogen synthase kinase 3
MCL-1	Myeloid Cell Leukemia 1
PCR	polymerase chain reaction
SCF	complex of SKP1, Cullin 1, F-box protein
SI-2	SRC-3 inhibitor-2
STR	short tandem repeat
RPMI	Roswell Park Memorial Institute medium
S	Synthesis phase of cell cycle
shRNA	short hairpin RNA
SRC-3	steroid receptor coactivator 3
TCGA	The Cancer Genome Atlas
WD repeat	tryptophan-aspartic acid dipeptide repeat

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Figure 1. Phosphorylated Cyclin E1, SRC-3, c-MYC, Rictor, GSK3, P70S6, and AKT protein levels increase in serous EC cells transiently expressing mutant FBXW7α compared to those expressing wildtype FBXW7α.

Western blot results of phosphorylated (P-) and total proteins detected in whole cell lysates of ARK1 (a, c) and ARK2 (b, d) serous EC cells collected 24hrs after transfection with 2.5μg of MYC-tagged vector control, wildtype (WT) or mutant *FBXW7α* expression constructs. Blots shown are representative of triplicate experiments.

Figure 2. Compared to parental ARK1 cells, CRISPR-edited *FBXW7*-mutant cells exhibit increased phosphorylated protein levels of Cyclin E1, SRC3, Rictor, GSK3, P70S6 and AKT proteins.

Western blot results of phosphorylated (P-) and total proteins detected in whole cell lysates of ARK1 serous EC cells collected 24hrs after plating. Blots shown are representative of triplicate experiments.

Figure 3. Compared to parental ARK1, CRISPR-edited *FBXW7*-mutant cells exhibit decreased viability and increased caspase 3/7 activity in response to SI-2. (a) Results of the Promega Cell Titer Glo Viability Assay 48hrs after treatment with 10, 25, 50, 100 and 200nM SI-2. Error bars represent standard deviation of triplicate experiments. Tables provide adjusted p-values calculated using a one-way ANOVA and Dunnett's multiple comparisons test and absolute IC50 values calculated using nonlinear regression fit of the inhibitor concentration versus the DMSO-normalized response using GraphPad Prism software; ns= not significant. (b) Results of the Promega Caspase Glo 3/7 Assay 24hrs after treatment with 25, 50, and 100nM SI-2. Error bars represent standard deviation of triplicate experiments. Table provide adjusted p-values calculated using a one-way ANOVA and Dunnett's multiple comparisons test.

[Dinaciclib]	Adjusted P-values- Parental vs. Each Mutant					
	G423V	R465C	R465H/ R465Afs*7	R479Q	R505C	R689W
5nM	0.9996 (ns)	0.6955 (ns)	0.3767 (ns)	0.0515 (ns)	0.9489 (ns)	0.9999 (ns)
10nM	0.6368 (ns)	0.0122	0.3207 (ns)	0.6257 (ns)	0.0885 (ns)	0.9879 (ns)
25nM	0.1804 (ns)	0.0018	0.0269	0.9176 (ns)	0.5625 (ns)	0.9996 (ns)
50nM	0.0003	0.0001	0.0001	0.0131	0.0027	0.1932 (ns)
100nM	0.0001	0.0001	0.0001	0.0021	0.0005	0.0074

Absolute IC50 (nM)						
Parental	G423V	R465C	R465H/ R465Afs*7	R479Q	R505C	R689W
9.6	8.6	8.4	8.3	8.7	8.6	9.3

Figure 4. Compared to parental ARK1, CRISPR-edited *FBXW7*-mutant cells exhibit decreased viability in response to dinaciclib.

Results of the Promega Cell Titer Glo Viability Assay 24hrs after treatment with 1, 5, 10, 25, 50, and 100nM dinaciclib. Error bars represent standard deviation of triplicate experiments. Tables provide adjusted p-values calculated using a one-way ANOVA and Dunnett's multiple comparisons test and absolute IC₅₀ values calculated using nonlinear regression fit of the inhibitor concentration versus the DMSO-normalized response using GraphPad Prism software; ns= not significant.

Figure 5. Dinaciclib treatment induced more apoptosis in CRISPR-edited *FBXW7*-mutant ARK1 cells than parental cells.

(a) Results of the Promega Caspase Glo 3/7 Assay 24hrs after treatment with 1, 10, and 100nM dinaciclib. Error bars represent standard deviation of quadruplicate experiments. Brackets indicate significant differences in caspase 3/7 activity in ARK1 parental cells versus CRISPR-edited *FBXW7*-mutated ARK1 cells calculated using a one-way ANOVA.

(b) Flow cytometry results using the Calbiochem Annexin V-FITC Apoptosis Detection Kit 24hrs after treatment with 10 and 100nM dinaciclib. Bars represent viable cells that did not stain with propidium iodide or annexin V-FITC (blue), cells in early apoptosis that only stained with annexin V-FITC (orange), and cells in late apoptosis that stained with both propidium iodide and annexin V-FITC (grey). Error bars represent standard deviation of triplicate experiments. Brackets and p-values indicate significant differences in Annexin V-

positive cells calculated using a one-way ANOVA and Dunnett's multiple comparisons test.
(c) Western blot results of pro-apoptotic and pro-survival proteins detected in whole cell lysates of ARK1 serous EC cells collected 24hrs after 100nM dinaciclib treatment. Blots shown are representative of triplicate experiments.

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Figure 6. Dinaciclib treatment caused changes in cell cycle distribution that differed between parental and CRISPR-edited *FBXW7*-mutant ARK1 cells.

(a) Flow cytometry results using the Exalpha Biologicals NUCYCL PI Kit 24hrs after treatment with 10 and 100nM dinaciclib. Bars represent cells the average percentage of cells in sub G1 (black), G1 (yellow), S (grey), G2/M (purple) and greater than G2/M (blue) phases of the cell cycle. Error bars represent standard deviation of triplicate experiments. Brackets and p-values indicate differences in cell cycle phases determined by a one-way ANOVA. (b) Western blot results of proteins detected in whole cell lysates of ARK1 serous EC cells collected 24hrs after 100nM dinaciclib treatment. Blots shown are representative of triplicate experiments.