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The Proapoptotic F-box Protein Fbxl7 Regulates Mitochondrial Function by Mediating the Ubiquitylation and Proteasomal Degradation of Survivin*

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Background: The SCF ubiquitin E3 ligase component Fbxl7 possesses proapoptotic activity.
Results: Fbxl7 targets the antiapoptotic protein survivin for polyubiquitylation and proteasomal degradation.
Conclusion: Survivin protects mitochondria from damage induced by Fbxl7.
Significance: Understanding how F-box proteins regulate survivin might impact therapies to preserve cellular bioenergetics.

Fbxl7, a component of the Skp1·Cul1·F-box protein type ubiquitin E3 ligase, regulates mitotic cell cycle progression. Here we demonstrate that overexpression of *Fbxl7* in lung epithelia decreases the protein abundance of survivin, a member of the inhibitor of apoptosis family. Fbxl7 mediates polyubiquitylation and proteasomal degradation of survivin by interacting with Glu-126 within its carboxyl-terminal α helix. Furthermore, both Lys-90 and Lys-91 within survivin serve as ubiquitin acceptor sites. Ectopically expressed *Fbxl7* impairs mitochondrial function, whereas depletion of Fbxl7 protects mitochondria from actions of carbonyl cyanide *m*-chlorophenylhydrazone, an inhibitor of oxidative phosphorylation. Compared with wild-type survivin, cellular expression of a survivin mutant protein deficient in its ability to interact with Fbxl7 (E126A) and a ubiquitylation-resistant double point mutant (KK90RR/KK91RR) rescued mitochondria to a larger extent from damage induced by overexpression of *Fbxl7*. Therefore, these data suggest that the Skp1·Cul1·F-box protein complex subunit Fbxl7 modulates mitochondrial function by controlling the cellular abundance of survivin. The results raise opportunities for F-box protein targeting to preserve mitochondrial function.

Apoptosis plays essential roles in organogenesis and as a natural process to remove damaged or abnormal cells that arise in the course of numerous biological events, such as recovery from inflammation or tissue injury. In mammalian systems, apoptosis occurs through two pathways: the extrinsic pathway, utilizing cell surface death receptors that recognize ligands, and the intrinsic pathway, including the mitochondrion as an essential component (1). In response to cellular stress, proapoptotic proteins translocate to the mitochondria, where they activate Bax and Bak, which trigger mitochondrial outer membrane permeabilization, which subsequently allows the release of cytochrome *c* and other mediators that induce caspase activation and ensuing apoptosis (2). Inhibitor of apoptosis (IAP)[4] proteins comprise a family of antiapoptotic proteins that bind to and neutralize proapoptotic proteins, interfere with the activation of caspases, and promote critical prosurvival signaling pathways (3). Survivin, the smallest member of the IAP protein family, plays multiple roles in cytoprotection and mitosis progression (4). Studies have revealed that survivin expression is up-regulated in human cancer specimens, which is linked to chemotherapeutic resistance and a poor prognosis (5–8). Additionally, survivin is involved in mitochondrial network remodeling by fission and fusion events, which allows mitochondria to adapt to cellular metabolic needs (9). On the basis of its essential roles in cell survival and mitosis (10), substantial attention has focused on factors that control survivin regulation at levels of transcription, posttranslational modification, and activity (11, 12). However, survivin protein stability, as an important indicator of protein abundance and function, remains relatively unexplored.

Cellular protein concentrations and subsequent biological function are largely controlled by the ubiquitin proteasome system. The ubiquitylation of a target protein in humans is carefully orchestrated by an enzymatic cascade including a ubiquitin-activating enzyme (E1), approximately one of 30 ubiquitin-conjugating enzymes (E2), and a ubiquitin E3 ligase, of which hundreds exist in cells. E3 ligases confer specificity to

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[4] The abbreviations used are: IAP, inhibitor of apoptosis; SCF, Skp1·Cul1·F-box; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MLE, murine lung epithelial; CHX, cycloheximide; BIR, baculoviral inhibitor of apoptosis repeat; CREB, cAMP response element-binding protein.

ubiquitylation by recruiting substrates and mediating the transfer of activated ubiquitin from an E2 to a substrate (13). Over 600 human genes encode RING domain-containing E3 ligases (14). Among these E3 ligases, the SCF superfamily modulates diverse biological processes (15). The SCF complex is composed of a catalytic core containing Skp1, Cul1, Rbx1, and an F-box protein. The small RING protein Rbx1 receives ubiquitin from an E2 enzyme, and the variable F-box protein recruits a substrate and bridges ubiquitin to the substrate (16). On the basis of the specific and precise regulations of SCF E3 ligases in eliminating substrates, SCF components have recently attracted significant attention in cancer therapeutic drug development (17, 18).

F-box proteins contain an NH_2-terminal 40-amino acid F-box domain that binds Skp1 to create a link to Cul1-Rbxl. On the basis of the carboxyl-terminal domain, F-box proteins are classified as an L subfamily (containing a leucine-rich repeat motif), a W subfamily (containing a WD repeat motif) and an O subfamily (F-box only) (19). F-box proteins recognize diverse substrates through the leucine-rich repeat/WD motif. Identifying the interaction between an F-box protein and its substrates has led to an improved understanding of how SCF E3 ligases mediate multiple biological functions, including gene expression, mitotic cell cycle progression, and inflammation. A recently characterized F-box protein, Fbxl7 has been shown to regulate mitosis through targeting Aurora A kinase for polyubiquitylation and proteasomal degradation, which plays vital roles in mitotic spindle formation and chromosome segregation (20). Our previous studies have demonstrated that Fbxl7 displays proapoptotic activity (20, 21). However, the nature of the molecular target for this F-box protein remained obscure. Because mitochondria play central roles in initiating apoptosis through the intrinsic pathway, we hypothesized that Fbxl7 mediates the disposal of an antiapoptotic protein that leads to impaired mitochondrial function. In a screening analysis, we identified that Fbxl7 recruits the antiapoptotic protein survivin for its polyubiquitylation and proteasomal degradation. Coexpression of *survivin* in cells largely protected mitochondria from injury induced by the ectopic overexpression of *Fbxl7*.

EXPERIMENTAL PROCEDURES

Cells—Murine lung epithelial (MLE12) cells (ATCC) and Beas-2B cells were cultured with HITES medium (DMEM:F12 supplemented with insulin, transferrin, hydrocortisone, β-estradiol and glutamine) containing 10% FBS and antibiotics as described previously (22). HeLa cells were cultured with EMEM medium (Eagle's Minimum Essential Medium) containing 10% FBS and antibiotics. HCT8 cells (ATCC) were cultured with RPMI 1640 medium containing 10% FBS and antibiotics.

Reagents—V5 antibody, the pcDNA3.1D/V5-His-TOPO cloning kit, and *Escherichia coli* Top10 One-Shot-competent cells were from Invitrogen. The PathScan stress and apoptosis signaling antibody Array kit, V5 rabbit antibody, Skp1 rabbit antibody, Cul1 rabbit antibody, and survivin rabbit antibody were from Cell Signaling Technology (Danvers, MA). Mfn1 rabbit antibody was from Abcam (Cambridge, MA). Leupeptin, β-actin mouse monoclonal antibody, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma. MG-132 was from UBPBio (Aurora, CO). Protease inhibitor tablets and Supersignal West Femto chemiluminescent substrate were from Thermo Scientific (Rockford, IL). The *Fbxl7* cDNA, *survivin* cDNA, scrambled shRNA, mouse *Fbxl7* shRNAs, and mouse *survivin* shRNAs were from OpenBiosystems (Huntsville, AL). Nucleofector transfection kits were from Amaxa (Gaithersburg, MD). QuikChange site-directed mutagenesis kits were from Agilent (Santa Clara, CA). Immobilized protein A/G beads were from Pierce. Fbxl7 rabbit antibody was from Novus (Littleton, CO). Purified Cul1, Rbx1, and Skp1 were from Abnova (Taipei, Taiwan). Purified ubiquitin, E1, E2, and ubiquitin aldehyde were from Enzo Life Sciences (Farmingdale, NY). Cycloheximide was from Calbiochem (La Jolla, CA). Fbxl7 mouse antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). TnT quick-coupled transcription/translation systems and the Enliten ATP assay system were from Promega (Madison, WI).

Cloning and Transfection—PCR-based approaches were applied to clone *Fbxl7* and survivin into pcDNA3.1/V5-HIS-TOPO vector (Invitrogen) for expression in mammalian cells using the appropriate primers. All mutants were constructed with PCR-based approaches or site-directed mutagenesis (Agilent). Plasmids were transfected into cells using nucleofection following the protocol of the manufacturer.

Flow Cytometry—Transfected cells were incubated with MitoTracker Red (50 nM) for 15 min following the protocols of the manufacturer (Invitrogen). Cells were washed with medium four times before harvesting with trypsin digestion. Cell suspensions were then analyzed with an AccuriC6 system with De Novo software. MitoTracker staining was measured by flow cytometry FL1 (FITC; excitation wavelength, 488 nm; emission wavelength, 530 nm) and FL3 (MitoTracker Red; excitation wavelength, 488 nm; emission wavelength, 610 nm). The enclosed areas represent the percentage of defective mitochondria.

Immunoblotting and Immunoprecipitation—Whole cell extracts (normalized to total protein concentration) were subjected to SDS-PAGE, electrotransferred to membranes, and immunoblotted. For immunoprecipitation, 1 mg of cell lysates (in PBS with 0.5% Triton X-100 plus protease inhibitors) were incubated with 2 μg of Fbxl7 rabbit antibodies for 3–4 h at 4 °C, followed by addition of 30 μl of protein A/G-agarose for an additional 1 h at 4 °C. The precipitated complex was washed three times with 0.5% Triton X-100 in PBS and analyzed by immunoblotting with an enhanced ECL system.

In Vitro Ubiquitylation Assays—The ubiquitylation of wild-type or lysine mutant survivin-HA was performed in a volume of 20 μl containing 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 0.6 mM DTT, 400 μM MG132, 2 mM ATP, 50 nM E1, 0.5 μM UbcH5, 0.5 μM UbcH7, 2 μM ubiquitin, 1 μM ubiquitin aldehyde, 20 nM Cul1, 20 nM Rbx1, 20 nM Skp1, *in vitro*-synthesized survivin-HA (WT or mutant), and Fbxl7 within the TnT-coupled reticulocyte lysate system. Reaction products were examined for HA immunoblotting.

Microscopy and Immunostaining—The microscopy analysis was performed using a Nikon A1 confocal microscope with a ×60 oil objective. The microscope was equipped with a Ti Perfect Focus system and Tokai Hit live cell chamber providing a humidified atmosphere at 37 °C with 5% CO_2. Transfected cells

were plated at 70% confluence on 35-mm MatTek glass bottom culture dishes before being labeled with MitoTracker Red (50 nM) or JC-1 (2 μM)for 20 min. Images were analyzed by Nikon NIS-element software. Pseudocolor green was used for optimal resolution of mitochondria MitoTracker image display.

Mitochondrial Bioenergetics—The indicated plasmids were nucleofected into MLE12 cells and incubated for 36 h before analysis. ATP was assayed using an Enliten ATP assay system (Promega). Oxygen consumption in cells was measured using an XF analyzer (Seahorse Biosciences). Oligomycin (3 μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.5 μM) and rotenone (3 μM) were added sequentially to assess non-ATP linked, maximal, and non-mitochondrial respiration, respectively.

Statistical Analysis—Statistical analysis was performed with an analysis of variance or unpaired Student's *t* test, with $p < 0.05$ indicative of significance.

RESULTS

Fbxl7 Modulates Mitochondrial Function—As we reported previously (20), ectopically expressed *Fbxl7* induces apoptosis in cells. To further decode the underlying mechanism, we tested whether Fbxl7 affected mitochondrial function. In Fig. 1*A*, flow cytometry-based MitoTracker staining revealed that the ectopically expressed plasmid encoding *Fbxl7* in MLE cells induces mitochondrial damage in a dose-dependent manner. Fbxl7 protein expression was examined by immunoblotting showing high levels in cells (Fig. 1*A*, *bottom panel*). We further compared mitochondrion morphology in cells transfected with either control empty vector or an *Fbxl7* plasmid. As a control, the mitochondrial membrane depolarizer CCCP induced formation of punctate-like signals of mitochondria instead of the reticular or more linear form observed in healthy control cells (Fig. 1*B*). Overexpressed Fbxl7 triggered mitochondrial changes comparable with CCCP treatment. Mitochondrial health is affected by the ability of these organelles to remodel through fusion. Mitochondrial fusion is mediated partly by the outer mitochondrial membrane fusion protein mitofusin 1 (Mfn1). CCCP treatment decreased Mfn1 protein levels in a dose-dependent manner (Fig. 1*C*). Compared with a control vector and an F-box domain-deficient *Fbxl7* plasmid that lacks the ability to form a SCF E3 ligase, overexpression of wild-type *Fbxl7* in cells reduced Mfn1 protein levels 36%, similar to the effects of CCCP treatment (Fig. 1*C*). To further link these mitochondrial structural changes induced by *Fbxl7* with a physiologic readout, we measured the membrane potential ($\Delta\Psi$) in *Fbxl7* overexpression cells using JC-1 (Fig. 1*D*). JC-1 dye displays potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Therefore, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence ratio. Similar to CCCP-treated cells, *Fbxl7*-overexpressed cells showed significant changes in membrane potential, indicative of bioenergetic changes in comparison with control cells. Additionally, increasing amounts of ectopically expressed *Fbxl7* also reduced ATP levels in cells, as shown in Fig. 1*E*. We further confirmed the effects of Fbxl7 on mitochondrial function in cells depleted of Fbxl7 (Fig. 1*F*). Compared with severe mitochondrial injury observed in cells exposed to CCCP and transfected with a control scrambled shRNA, Fbxl7 depletion in cells largely protected mitochondria from mitochondrial membrane depolarization induced by CCCP (Fig. 1*F*). These results suggest that Fbxl7 dose-dependently induces mitochondrial dysfunction.

As a component of the SCF type ubiquitin E3 ligase, Fbxl7 recruits substrates for ubiquitylation and degradation. To interpret the mechanism underlying the Fbxl7 adverse effects on mitochondrial function, we performed a substrate screening assay after overexpressing the *Fbxl7* plasmid in cells and measuring stress- and apoptosis-signaling substrates. We identified that high Fbxl7 protein levels were sufficient to decrease survivin protein abundance but had no effects on other mitochondrial proteins, such as Bad or caspase 3 (data not shown). Because survivin affects the fission of mitochondria (9), we next investigated how this protein affects mitochondrial function.

Survivin Knockdown Induces Mitochondrial Deficiency—We first screened survivin shRNA knockdown efficiency for functional studies (Fig. 2*A*). Survivin shRNA 1 and 4 sufficiently decreased the protein levels of survivin. However, these shRNAs had no effect on Cul1 and Skp1 protein abundance. JC-1 staining demonstrated that effective knockdown of survivin significantly increased the green/red fluorescence ratio compared with the effects of shRNAs that exhibited a limited ability to deplete survivin (*survivin* shRNAs *2*, *3*, and *5*; Fig. 2*B*). Fig. 2*C* indicates that knockdown of survivin significantly decreased the oxygen consumption rate of cells, suggesting that survivin knockdown injures the capacity of mitochondrial respiration. MitoTracker staining combined with flow cytometric measurements demonstrated that survivin knockdown, compared with scrambled shRNA, results in mitochondrial impairment (Fig. 2*D*). Compared with cells transfected with control scrambled shRNA, the cells depleted with survivin displayed distorted punctate-like mitochondria (Fig. 2*E*). These results show that knockdown of survivin displays a mitochondrial phenotype similar to the ectopically expressed *Fbxl7* plasmid in cells. The results led us to investigate how Fbxl7 targets survivin for its elimination in cells.

Fbxl7 Mediates the Polyubiquitylation and Proteasomal Degradation of Survivin—To better characterize survivin protein, we first measured the half-life of endogenous survivin protein in lung epithelial cells. As shown in Fig. 3*A*, cells treated with the protein synthesis inhibitor cycloheximide (CHX) indicated that the half-life of survivin is ~2 h. Treatment with the proteasome inhibitor MG132 largely stabilized the decay of survivin. However, the lysosome inhibitor leupeptin did not modulate survivin degradation kinetics. These results suggest that the proteasome, rather than the lysosome, is involved in degradation of survivin. We further measured whether Fbxl7 mediates the ubiquitylation and proteasomal degradation of survivin. In *in vitro* ubiquitylation assays, as shown in Fig. 3*B*, the ubiquitin E3 ligase SCF^{Fbxl7} catalyzed the polyubiquitylation of survivin. Furthermore, the overexpressed *Fbxl7* plasmid in cells accelerated the degradation of endogenous survivin over time. However, overexpression of an Fbxl7 F-box deletion mutant had no effect on the half-life of endogenous survivin protein (Fig. 3*C*). Additionally, increasing amounts of ectopically

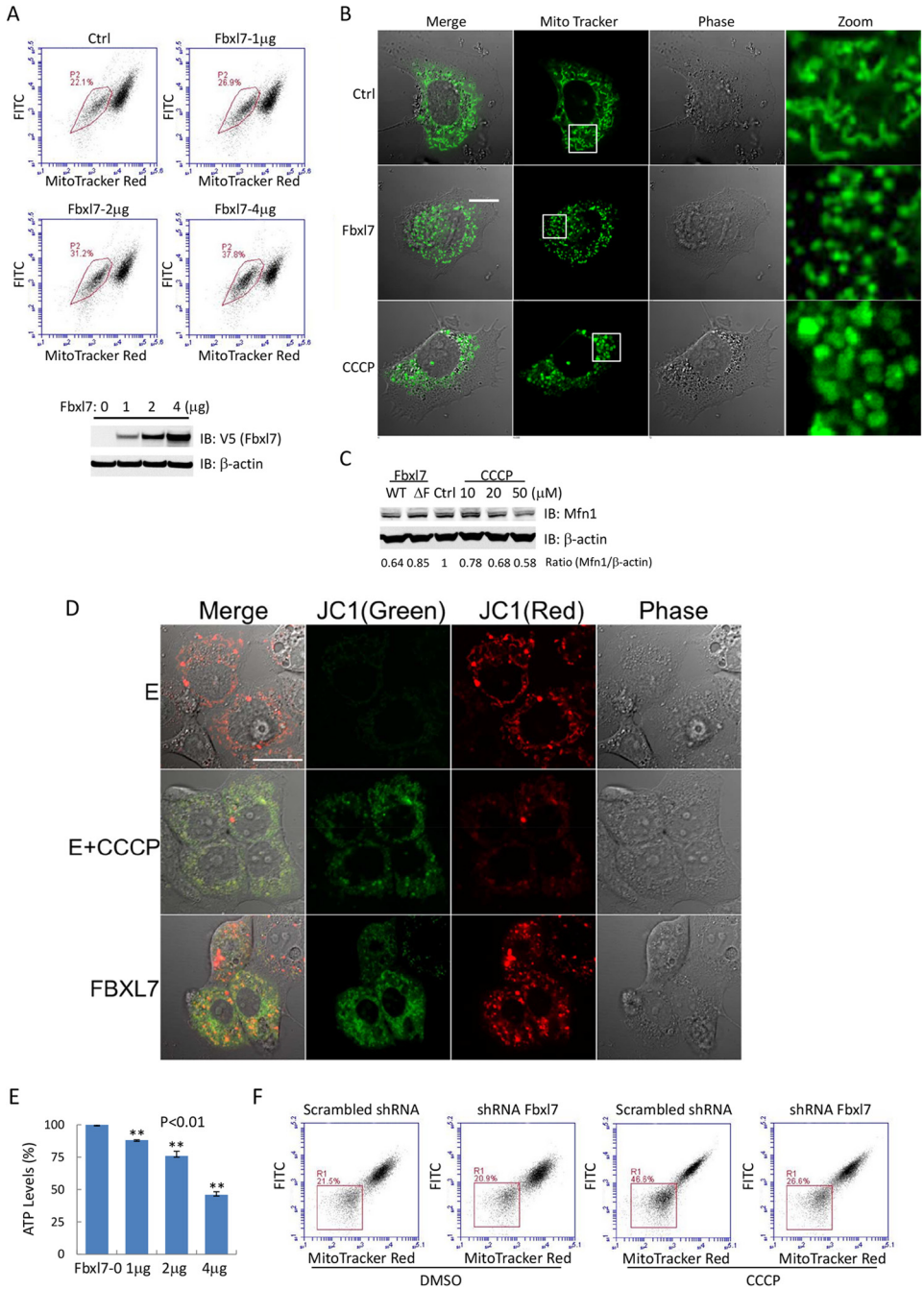

FIGURE 1. **Fbxl7 overexpression induces mitochondrial injury.** *A*, MLE12 cells were nucleofected with empty vector (*Ctrl*) or the indicated amounts of *Fbxl7* plasmid and split into two identical wells in 6-well plates. After incubation for 36 h, one group of cells was stained with MitoTracker Red (50 nM) for 20 min prior to analysis by flow cytometry. The *y*-axis FITC represents FL1 (excitation (*Ex*) wavelength, 488 nm; emission wavelength, 530 nm), and the *x*-axis MitoTracker Red represents FL3 (excitation wavelength, 488 nm; emission wavelength, 610 nm). The *enclosed areas* represent the percentage of defective mitochondria. The other identical group of cells was lysed and subjected to immunoblotting (*IB*) with V5 and β-actin antibodies, respectively. *B*, cells were nucleofected with 4 μg of empty vector (*Ctrl*) or *Fbxl7* plasmid and plated on 35-mm MatTek glass-bottom culture dishes. 48 h later, cells were treated with or without 20 μM CCCP for 1 h before the cells were stained with MitoTracker Red (50 nM) for 20 min before confocal microscopy analysis. Images were then obtained using pseudocolor green for MitoTracker image displays. *Scale bar* = 10 μm. *C*, MLE12 cells were nucleofected with empty vector, *Fbxl7*, or an *Fbxl7* F-box deletion mutant plasmid. After incubation for 46 h, cells with empty vector were treated with CCCP (10–50 μM) for an additional 2 h before analysis by Mfn1 and β-actin immunoblotting. Shown below are densitometric ratios from the quantitation of bands on immunoblots of Mfn1/actin. *D*, MLE12 cells were nucleofected with empty vector (*E*) or *Fbxl7* plasmid for 48 h. Cells were also treated with CCCP (50 μM) for 5 min before staining with JC-1 (2 μM) for 20 min. Cells were washed with warm PBS five times before analysis using confocal microscopy. *Scale bar* = 10 μm. *E*, cells transfected with the indicated amounts of *Fbxl7* plasmid were lysed by 2% TCA trichloroacetic acid and then neutralized in 20 mM Tris acetate (pH 7.75). ATP levels in the cell lysates were measured by an Enliten ATP assay kit, and the luminescence signal was analyzed in a BioTek Synergy 4 hybrid microplate reader and Gen5 2.00 software. Cellular ATP levels were normalized to Fbxl7 0 cells ($n = 3$; **, $p < 0.01$, indicative of significance *versus* Fbxl7 0). *F*, cells were transfected with scrambled shRNA control or *Fbxl7* shRNA. After incubation for 48 h, cells were treated with dimethyl sulfoxide (*DMSO*) or CCCP (20 μM) for 2 h before labeling with MitoTracker Red for flow cytometry. The *y*-axis FITC represents FL1 (excitation wavelength, 488 nm; emission wavelength, 530 nm), and the *x*-axis MitoTracker Red represents FL3 (excitation (*Ex*) wavelength, 488 nm; emission wavelength, 610 nm). The *enclosed areas* represent the percentage of defective mitochondria.

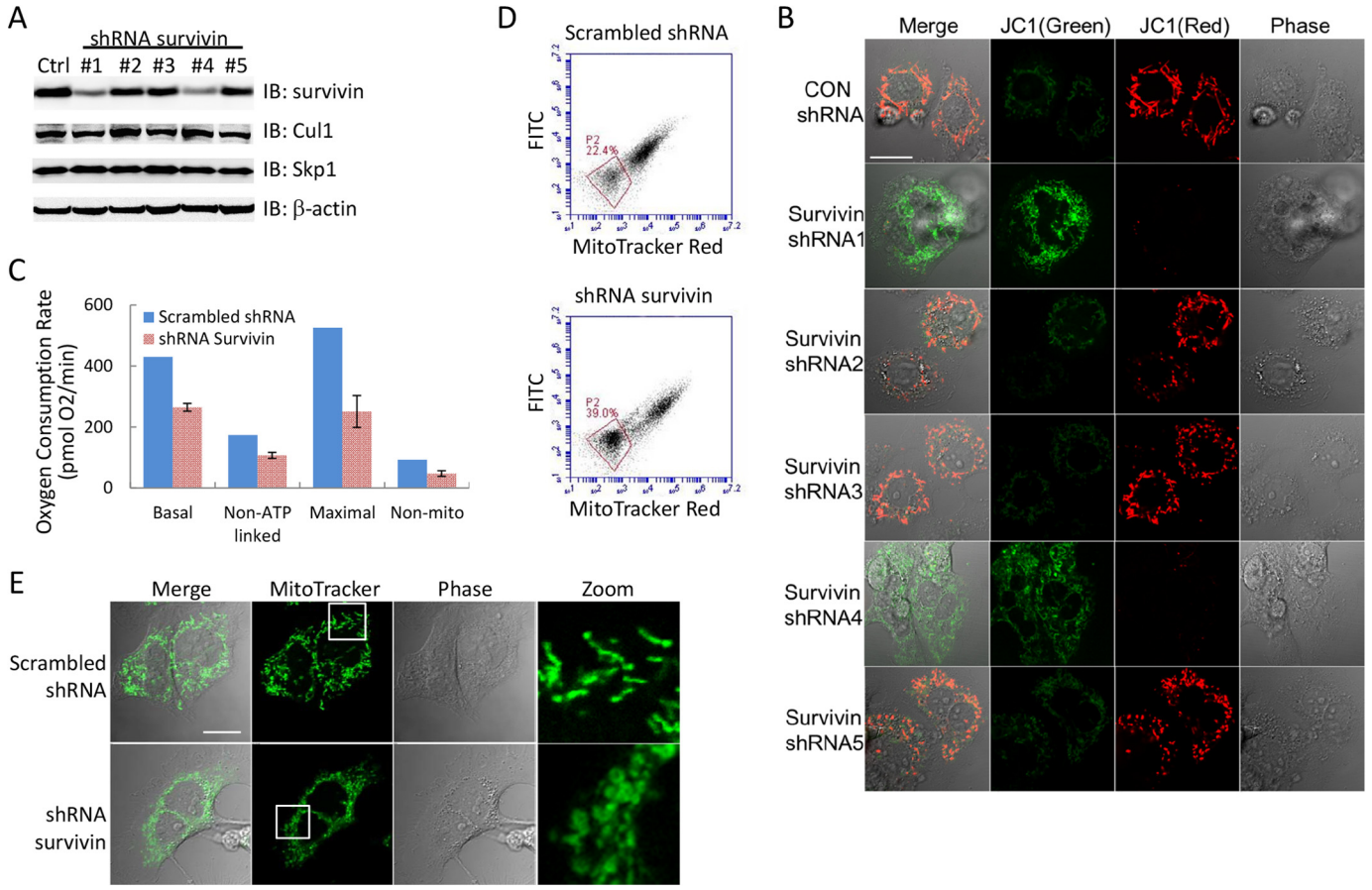

FIGURE 2. **Knockdown of survivin triggers mitochondrial defects.** *A*, MLE12 cells were nucleofected with scrambled shRNA (*Ctrl*) or one of several survivin shRNAs. After 48-h incubation, cell lysates were subjected to immunoblotting (*IB*) with either survivin, Cul1, Skp1, or β-actin antibodies. *B*, MLE12 cells were nucleofected with a control (*CON*) scrambled shRNA or one of several different survivin shRNAs for 48 h. Cells were stained with JC-1 (2 μM) for 20 min. Cells were then washed with warm PBS five times before analysis using confocal microscopy. *C*, cells transfected with scrambled shRNA or *survivin* shRNA 1 were subjected to a seahorse XF analyzer for oxygen consumption rate measurements. *Non-mito*, non-mitochondrial respiration. *D*, cells transfected with scrambled shRNA or *survivin* shRNA 1 were labeled with MitoTracker Red (50 nM) for 20 min prior to flow cytometry analysis. The *y*-axis FITC represents FL1 (excitation (*Ex*) wavelength, 488 nm; emission wavelength, 530 nm), and the *x*-axis MitoTracker Red represents FL3 (excitation wavelength, 488 nm; emission wavelength, 610 nm). The *enclosed areas* represent the percentage of defective mitochondria. *E*, cells were transfected with control scrambled shRNA or *survivin* shRNA 1. After incubation for 48 h, cells were labeled with MitoTracker for confocal microscopy analysis. *Scale bar* = 10 μm.

expressed *Fbxl7* plasmid resulted in a decrease in survivin protein levels (Fig. 3*D*). However, other randomly selected F-box proteins, Fbxo16 and Fbxo17, had no effects on survivin protein abundance. Furthermore, ectopic expression of *Fbxl7* also reduced survivin protein levels in other cell lines of epithelial origin (colon, cervical, and human airway cells), suggesting that the behavior of this F-box protein is not restricted to lung epithelia (Fig. 3*E*). To further confirm that Fbxl7 down-regulates survivin protein abundance, we screened a group of Fbxl7 shRNAs. Fig. 3*F* suggests that effective knockdown of Fbxl7 largely accumulates endogenous survivin protein. However the depletion of Fbxl7 had little influence on the proteins Cul1 and Skp1. Additionally, knockdown of Fbxl7 extended the lifespan of endogenous survivin (Fig. 3, *G* and *H*). As a whole, these data indicate that Fbxl7 mediates the polyubiquitylation and proteasomal degradation of survivin.

E126 within Survivin Serves as the Docking Site for Fbxl7—F-box proteins are recruited to their substrates using distinct molecular signatures. To dissect the Fbxl7 docking site with survivin, we constructed a series of amino- and carboxyl-truncated survivin expression plasmids, as shown in Fig. 4*A*. Survivin C-terminal deletion mutants displayed obvious binding deficiencies to Fbxl7 in coimmunoprecipitation assays. The most distal C-terminal 22 amino acids are important for Fbxl7 binding because, in pulldowns, survivin mutants (CD22–62) largely failed to interact with Fbxl7 (Fig. 4*A*, *bottom panel*, *top blot*). We then focused on this stretch of C-terminal residues within survivin to further narrow down the Fbxl7-interacting site, demonstrating that progressive loss of the 17–22 carboxyl-terminal residues resulted in reduced Fbxl7 binding, suggesting that these residues were required for optimal interaction between the F-box protein and survivin (Fig. 4*B*). Specifically, glutamic acid 126 within survivin was required for Fbxl7 binding (Fig. 4*C*). In protein half-life assays, the expression of plasmids encoding *WT*, *E126A*, or *T127A* survivin mutants was tested for stability (Fig. 4*D*), and densitometric analysis was performed (Fig. 4*E*). We confirmed that the Fbxl7 binding-defective E126A survivin mutant protein was more stable than the other constructs. These results show that the Glu-126 site functions as a key molecular recognition site for Fbxl7 binding.

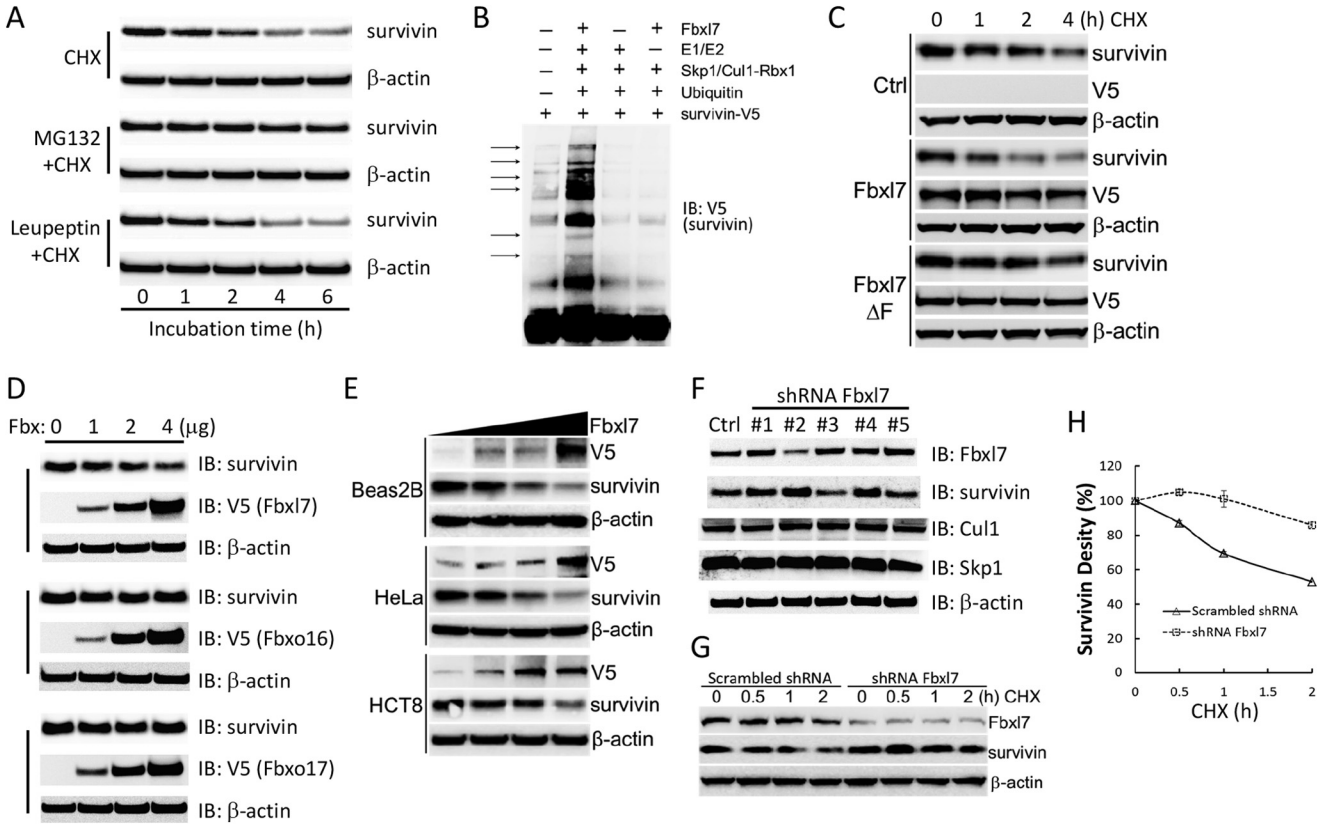

FIGURE 3. **Fbxl7 targets survivin for polyubiquitylation and proteasomal degradation.** *A*, MLE12 cells were treated with 20 μg/ml CHX for the indicated times with or without 1 h pretreatment with MG-132 (20 μM) or leupeptin (100 μM). Cell lysates were subjected to survivin and β-actin immunoblotting. *B*, *in vitro* ubiquitylation assays. A 20-μl reaction containing 50 nM ubiquitin-activating enzyme, 0.5 μM UbcH5, 0.5 μM UbcH7, 2 μM ubiquitin, 1 μM ubiquitin aldehyde, 20 nM Cul1, 20 nM Rbx1, 20 nM Skp1, *in vitro* synthesized survivin-V5, and Fbxl7 within the TnT coupled reticulocyte system. The reaction products were subjected to V5 immunoblotting (*IB*). *C*, survivin protein half-life was determined with overexpression of *Fbxl7* or the *Fbxl7* F-box deletion mutant. Cells transfected with 3 μg of empty vector (*Ctrl*), *Fbxl7*-V5, or *Fbxl7* F-box deletion-V5 plasmid were subjected to CHX treatment as indicated time point, and the cell lysates were processed for survivin, V5, and β-actin immunoblotting. *D*, MLE12 cells were transfected with the indicated amounts of *Fbxl7*-V5, *Fbxo16*-V5, or *Fbxo17*-V5, and the cell lysates were analyzed by survivin, V5, and β-actin immunoblotting. *E*, Beas-2B, HeLa, or HCT8 cells were transfected with increasing amounts of *Fbxl7*-V5 plasmid. After incubation for 48 h, the cell lysates were subjected to immunoblot analysis with V5, survivin, and β-actin antibodies. *F*, cells were nucleofected with either scrambled shRNA or *Fbxl7* shRNA clones. Endogenous Fbxl7, survivin, Cul1, Skp1, and β-actin were detected by immunoblotting. *G*, cells were nucleofected with either scrambled shRNA or shRNA Fbxl7–2 (5 μg). After incubation for 72 h, the cells were subjected to CHX treatment as indicated for various time points, and the cell lysates were processed for Fbxl7, survivin, and β-actin immunoblotting. *H*, the densitometry results of the survivin immunoblots were plotted for half-life analysis. Graphical data are representative of two independent experiments.

Lys-90 and Lys-91 within Survivin Are Required for Ubiquitin Ligation—To identify the ubiquitin acceptor site within survivin, we ectopically expressed *survivin* plasmids encoding point mutations at one or two lysine sites within the protein in cells. However, as shown in Fig. 5*A*, with CHX treatment, none of the single lysine mutants could stabilize the decay of survivin. On the basis of the crystal structure of survivin (PDB code 1F3H), we further constructed a series of survivin double lysine mutants. Specifically, to exclude the influence of another protease, we coexpressed *Fbxl7* and a *survivin* lysine mutant in cells to compare the protein levels in cells coexpressed with control empty vector *versus* a lysine mutant. Fig. 5*B* demonstrates that a dual lysine mutant, K90R/K91R, was significantly stabilized against the Fbxl7-mediated degradation of survivin. To further rule out potential effects of a V5 tag (containing a lysine), we also tested the survivin turnover rate with the lysine-free HA tag. As shown in Fig. 5*C*, a double lysine mutant, K90R/K91R, largely exhibited delayed degradation compared with the wild type or the other dual lysine mutant, K129R/K130R. The results were quantitated and are shown in Fig. 5*D*. The *in vitro* ubiquitylation assays shown in Fig. 5*E* confirmed that a double lysine mutant, K90R/K91R, displayed resistance to Fbxl7-dependent ubiquitylation. However, as shown in Fig. 5*F*, the survivin double lysine mutations had no effect on the interaction between Fbxl7 and survivin. These results suggest that both Lys-90 and Lys-91 are critical for Fbxl7-mediated polyubiquitylation.

Survivin Overexpression Attenuates Fbxl7-induced Mitochondrial Injury—In cells overexpressing *Fbxl7*, MitoTracker staining shows that the percentage of defective mitochondria increases significantly (Figs. 1 and 6*A*), as quantified in Fig. 6*B*. However, coexpression of wild-type *survivin* together with *Fbxl7* or an Fbxl7 survivin binding mutant (*E126A*) partially rescues the mitochondrial defect induced by the F-box protein (Fig. 6*B*). We also observed that expression of the ubiquitylation-resistant survivin dual lysine mutant *K90R/K91R* protects mitochondria from overexpressed *Fbxl7* effects to a larger extent compared with wild-type survivin. We also measured the ATP levels in cells transfected with these plasmids. Consistent with our observations on the basis of MitoTracker staining, *Fbxl7* overexpression caused a significant decrease in cellular ATP levels. However, co-overexpression of survivin largely counteracts these adverse effects on energy stores in cells over-

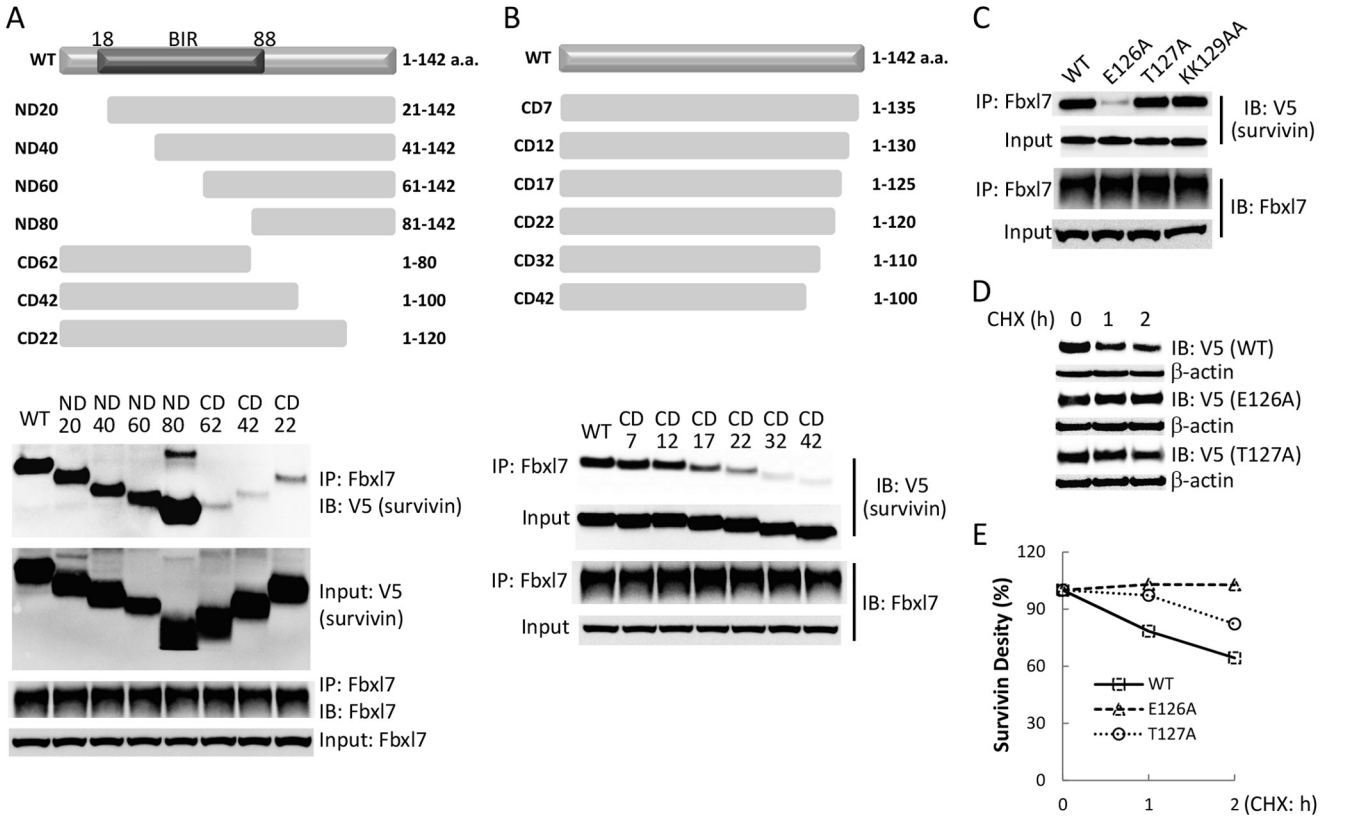

FIGURE 4. **Fbxl7 associates with the C-terminal α-helix within survivin.** *A* and *B*, truncated survivin mutants. *a.a.*, amino acids; *IP*, immunoprecipitation; *IB*, immunoblot. *A–C*, survivin-V5 proteins, including WT, truncates, and point mutants, were synthesized using a TnT coupled reticulocyte lysate system. MLE12 cell lysates were subjected to immunoprecipitation with Fbxl7 antibody and protein A/G-agarose. We synthesized either wild type or survivin mutants *in vitro* that were then subjected to coimmunoprecipitation with Fbxl7 beads. The precipitates were eluted for immunoblotting with Fbxl7 and V5 antibodies. *ND*, N-terminal deletion; *CD*, C-terminal deletion. *D*, survivin protein half-life was determined in cells transfected with V5 tagged survivin wild-type or point mutant plasmids. The immunoblots were analyzed by V5 and β-actin antibodies. Each protein band on the immunoblots was quantified densitometrically and is shown graphically in *E*.

expressing *Fbxl7*. Specifically, expression of a degradation-resistant survivin lysine mutant displayed a greater degree of protection against injurious effects of *Fbxl7* overexpression cells compared with wild-type survivin (Fig. 6*C*). To specifically monitor mitochondrial function further, we measured the oxygen consumption rate with a seahorse XF analyzer. As shown in Fig. 6*D*, the mitochondrial oxygen consumption rate decreased significantly in *Fbxl7*-overexpressed cells. Additional exogenous expression of *survivin* compensated for the mitochondrial respiration defect induced by *Fbxl7* overexpression. An Fbxl7 binding-deficient survivin plasmid (*E126A*) mutant, when expressed in cells, also displayed a greater capacity than wild-type survivin to rescue the respiratory deficiency triggered by overexpression of *Fbxl7*. These results underscore the importance of survivin protein stability within cells to maintain mitochondrial bioenergetics.

DISCUSSION

The results shown here demonstrate that survivin is regulated at the level of protein stability through its site-specific interaction and ubiquitylation by the SCF^{Fbxl7} complex. The abundance of survivin is essential to maintain mitochondrial integrity and function because depletion of this protein, either genetically or mediated by the F-box protein Fbxl7, results in impaired mitochondrial structure and reduced ability of these organelles to generate chemical energy. Whether Fbxl7 is activated during pathobiological conditions in humans requires additional studies. Nevertheless, the results of these studies might be important in the design, synthesis, and testing of small-molecule antagonists to F-box subunits that might be injurious through activation of cell death effector pathways. Such small-molecule-targeted therapeutics are already in preclinical testing (23–28).

The IAP proteins belong to a family of proteins that are involved in cell cycle progression, cell death, immunity, and inflammation (3). The human IAP family includes the proteins: BIRC1, c-IAP1 (BIRC2), c-IAP2 (BIRC3), X-linked inhibitor of apoptosis (BIRC4), survivin (BIRC5), Apollon/Bruce (BIRC6), (BIRC7), and ILP-2 (BIRC8). The members of this protein family contain one to three baculoviral IAP repeats (BIR) domains, an ~70 amino acid zinc-binding domain that was first identified through sequence homology among proteins belonging to the IAP family (29). Apart from the BIR domain, several members of this family also possess a ubiquitin associated domain and a RING domain, which provides these proteins with ubiquitin E3 ligase activity (30). However, survivin is only composed of one NH_2-terminal BIR domain and a C-terminal coiled helix. On the basis of our binding results, the C-terminal amphipathic α helix of survivin is important

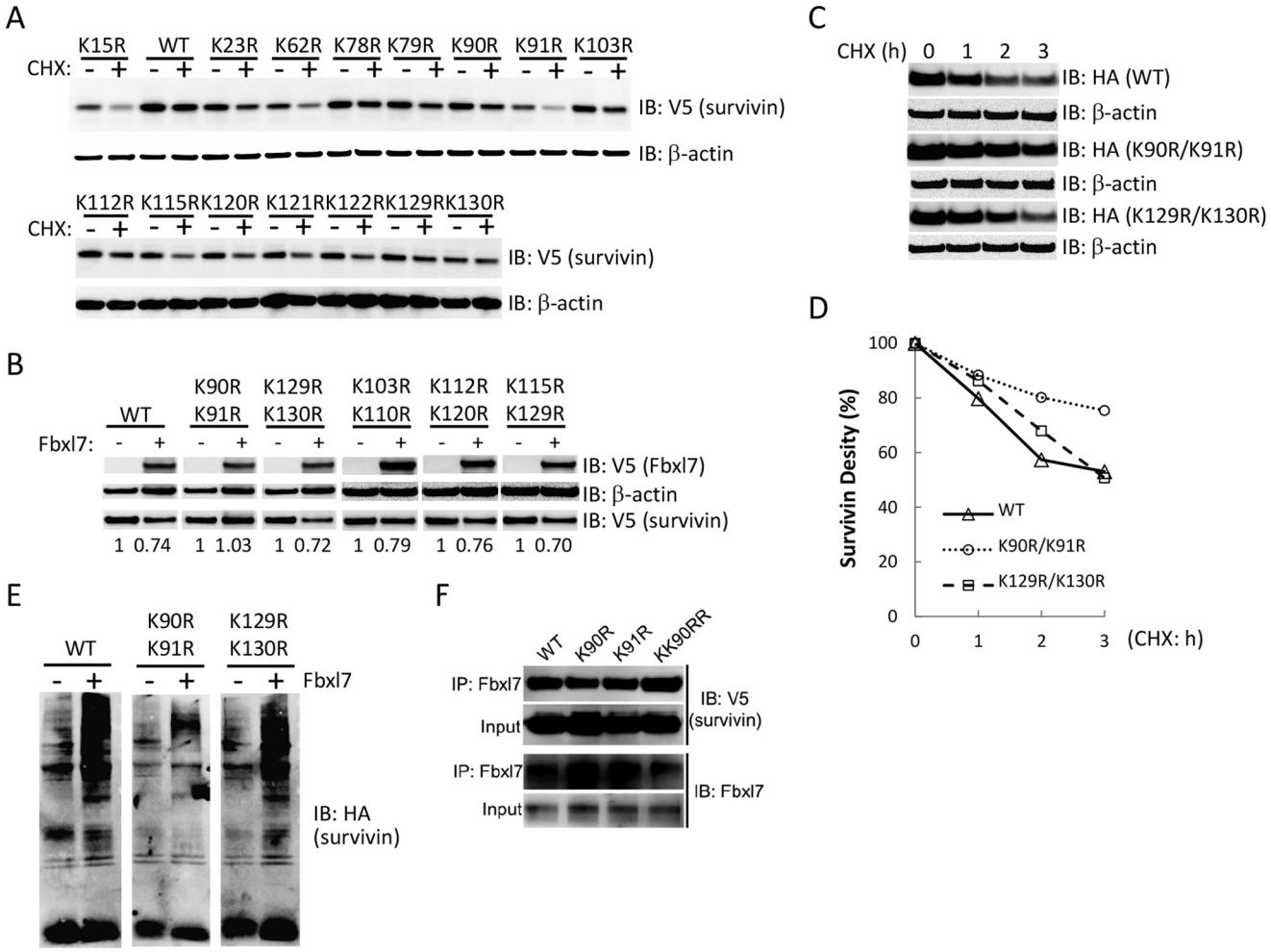

FIGURE 5. **Both Lys-90 and Lys-91 are required for Fbxl7-dependent ubiquitin docking within survivin.** *A*, each V5-tagged *survivin* single lysine protein mutant or WT *survivin* plasmid was ectopically expressed in MLE12 cells with or without 20 μg/ml CHX treatment for 2 h. Cell lysates were subjected to V5 and β-actin immunoblotting (*IB*). *B*, each V5-tagged *survivin* dual lysine mutant plasmid was coexpressed with empty vector or *Fbxl7*-V5 plasmid. Both survivin and Fbxl7 protein levels were measured by V5 immunoblotting with β-actin as a loading control. Shown below are densitometric values from the quantitation of bands on V5 immunoblots. *C*, the half-life of survivin was determined in cells transfected with HA-tagged *survivin* wild-type or dual lysine mutants. Immunoblots were analyzed by HA and β-actin antibodies. Each protein band on the immunoblots was quantified densitometrically and is shown graphically in *D*. *E*, *in vitro* ubiquitylation assays. Similar to Fig. 3*B*, each reaction contained HA-tagged survivin wild-type or a dual lysine mutant as a substrate with or without Fbxl7 as the functional ligase. Polyubiquitylated survivin was detected using a HA antibody. *F*, similar to Fig. 4, *A–C*, TNT *in vitro* synthesized survivin-V5 proteins, including WT and point mutants, were subjected to coimmunoprecipitation (*IP*) with Fbxl7 beads. The precipitates were eluted for immunoblotting with Fbxl7 and V5 antibodies.

for Fbxl7 docking, which is consistent with its structural analysis, suggesting that the C-terminal coiled helix is an authentic protein-protein interaction region (31). In our current model, survivin Glu-126 might play a central role in maintaining the α helix that facilitates the recognition and interaction with Fbxl7.

We have reported previously that the F-box protein Fbxl7 possesses proapoptotic activity and that it targets the mitotic kinase Aurora A for polyubiquitylation and degradation (20). Survivin, similar to Aurora A, has multiple functions, including inhibiting apoptosis and regulating mitosis. The subcellular localization of Fbxl7 and survivin is likely critical for their specified function. Although here we mainly focused on the biochemical features of how Fbxl7 targets and regulates survivin protein concentrations in lung cells and the F-box protein ability to modulate mitochondrial function, future studies will need to ascertain several modes of control. For example, how Fbxl7-survivin dynamics interplay with cell cycle progression and cross-talk between mitotic events in the nucleus with mitochondrial activity may be important.

The ability of F-box proteins to recognize their substrates must be precisely controlled. In response to various stimuli, F-box proteins must recruit substrates rapidly and specifically to the catalytic core for ubiquitylation. Phosphorylation of the substrate generates a canonical phosphodegron for F-box protein binding that either speeds up or slows down substrate degradation (32, 33). F-box proteins also recognize glycosylated substrates through F-box-associated domains (34, 35). Acetylation has been recognized recently as an important posttranslational modification to regulate protein stability (36). In the C-terminal amphipathic region, survivin undergoes Aurora B-dependent phosphorylation on Thr-117 (37) and CREB-binding protein-mediated acetylation on Lys-129 (38). The phosphomimetic T117E survivin mutant is unable to localize correctly during mitosis and exhibits an impaired ability to interact with inner centromere protein, chromosomal passen-

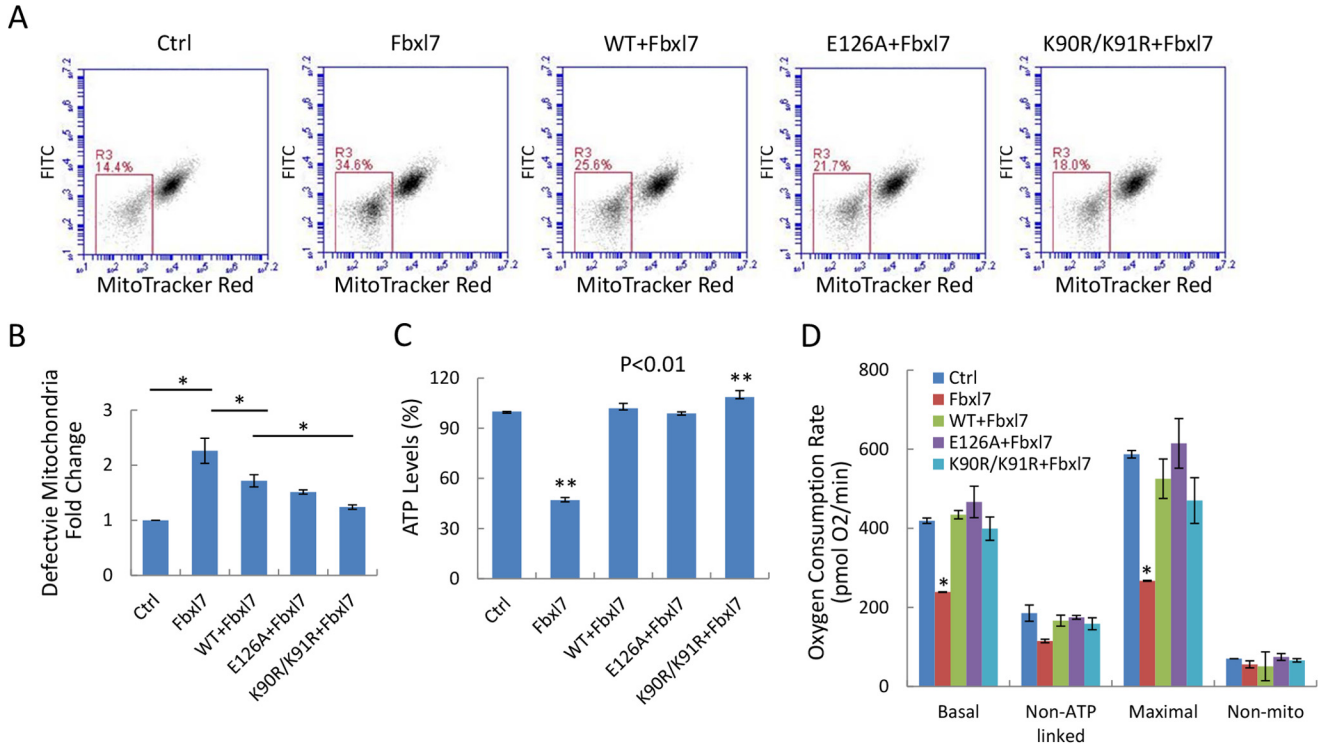

FIGURE 6. **Survivin and Fbxl7 synergistically modulate mitochondrial function.** *A*, MLE12 cells were transfected with empty vector (*Ctrl*), *Fbxl7*-V5 plasmid, or both *survivin* (WT or mutant)-V5 and *Fbxl7*-V5 plasmids. After 48-h incubation, cells were stained with MitoTracker Red (50 nM) for 20 min before flow cytometry analysis. The *y*-axis FITC represents FL1 (excitation (*Ex*) wavelength, 488 nm; emission wavelength, 530 nm), and the *x*-axis MitoTracker Red represents FL3 (excitation wavelength, 488 nm; emission wavelength, 610 nm). The *enclosed areas* represent the percentage of defective mitochondria. The defective mitochondrial percentage was normalized to the control group and is quantified in *B* ($n = 3$; data are mean ± S.E.; *, $p < 0.05$ is indicative of statistical significance between the control group and Fbxl7, Fbxl7 with or without WT survivin, or Fbxl7 with WT or K90R/K91R mutant survivin). *C*, MLE12 cells expressing the indicated plasmids were lysed and subjected to an Enliten ATP assay system. The cellular ATP levels were normalized to empty vector (*Ctrl*). Data represent three independent experiments (mean ± S.E.). **, $p < 0.01$, indicating statistical significance *versus* control or Fbxl7. *D*, MLE12 cells transfected with the indicated plasmids were subjected to a Seahorse XF analyzer for oxygen consumption rate measurements. Data represent three independent experiments (mean ± S.E.). *, $p < 0.05$, indicating statistical significance *versus* control. *Non-mito*, non-mitochondrial respiration.

ger, which disrupts mitotic cell cycle progression (37). The acetylation on Lys-129 directs survivin nuclear localization, and nuclear survivin binds to STAT3 and inhibits its transactivation activity (38). Therefore, an important area of study is to assess the impact of these posttranslational modifications that alter the subcellular localization on Fbxl7-mediated survivin ubiquitylation and degradation. The identity of a potential kinase or acetylase involved in modulating the interaction of Fbxl7 with survivin that affects the abundance of these prosurvival regulators is currently under investigation.

It has been reported that X-linked inhibitor of apoptosis-associated factor 1 (XAF1) recruits survivin for ubiquitylation and proteasomal degradation (12). The cytoplasmic Cul9 has also been newly identified as an E3 ligase for survivin degradation, and it can be inhibited by Cul7 (11). Our identification that Fbxl7 targets survivin further increases the complexity of IAP protein stability regulation. Three pathways might work within different cellular compartments to coordinate cell cycle phases, mitochondrial homeostasis, and cell survival in response to distinct signaling.

Cytoprotective and mitotic events executed by survivin favor cancer survival. In clinical specimens, survivin protein is highly expressed in human cancers that are linked to resistance to chemotherapy or radiation therapy with poor outcomes. Therefore, survivin has been proposed as an attractive target for small-molecule therapeutic targeting in neoplasia. Some pan-selective IAP antagonists targeting all IAPs are in phase I clinical trials (39–41). As an endogenous suppressor of survivin, Fbxl7 may serve as a unique target for pharmaceutical development. Specifically, a new chemical entity that facilitates the interaction of Fbxl7 with survivin would, theoretically, be predicted to accelerate the degradation of survivin. Conversely, on the basis of Fbxl7 knockdown protecting mitochondria from depolarization damage, developing an Fbxl7 inhibitor might be a promising strategy for metabolic disorders linked to mitochondrial dysfunction from survivin deficiency. The ability to up-regulate survivin levels in cells may also overcome deficiencies in other key elements that occur with processes where mitochondrial proteins are altered. Therefore, the strategy of optimizing expression of survivin protein by blocking Fbxl7 during mitochondrial fission, fusion, or mitophagy would be of interest.

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