**MFF REGULATION OF MITOCHONDRIAL CELL DEATH IS A THERAPEUTIC TARGET IN CANCER**

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**SUPPLEMENTARY MATERIAL**

Supplementary Table S1-S3

Supplementary Figs. S1-S6

**Supplementary Table S1**. MFF1 synthetic peptides used in this study.

|  |  |  |
| --- | --- | --- |
| **Peptide #** | **Sequence** | **Amino acid range** |
| **1** | AEMAEISRIQYEMEYTEGISQRMRVP | 25-50 |
| **2** | VPEKLKVAPPNADLEQGFQEGVPNASVIMQ | 49-78 |
| **3** | MQVPERIVVAGNNEDVSFSRPADLDLIQST | 77-106 |
| **4** | STPFKPLALKTPPRVLTLSERPLDFLDLER | 105-134 |
| **5** | ERPPTTPQNEEIRAVGRLKRERSMSENAVR | 133-162 |
| **6** | VRQNGQLVRNDSLWHRSDSAPRNKISRFQA | 161-190 |
| **7** | QAPISAPEYTVTPSPQQARVCPPHMLPEDG | 189-218 |
| **8** | DGANLSSARGILSLIQSSTRRAYQQILDVL | 217-246 |
| **9** | VLDENRRPVLRGGSAAATSNPHHDNVRYGI | 245-274 |
| **10** | GISNIDTTIEGTSDDLTV | 273-290 |
| **11** | VVDAASLRRQIIKLNRRLQLLEEENKERAKREM | 290-322 |

**Supplementary Table S2**. Mutant peptides derived from MFF1 peptide #8 used in this study.

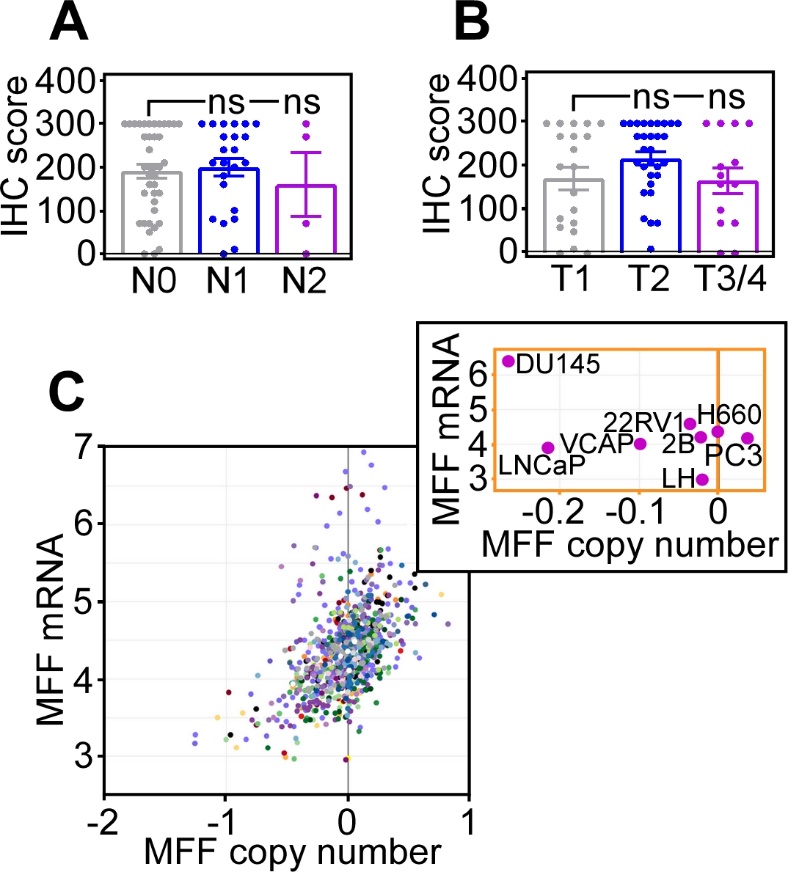
|  |  |
| --- | --- |
| **Peptide #** | **Sequence** |
| **8-1** | NLSSARGILSLIQSSTRRAYQQILDVL |
| **8-2** | SARGILSLIQSSTRRAYQQILDVL |
| **8-3** | GILSLIQSSTRRAYQQILDVL |
| **8-4** | SLIQSSTRRAYQQILDVL |
| **8-5** | QSSTRRAYQQILDVL |
| **8-6** | DGANLSSARGILSLIQSSTRRAYQQIL |
| **8-7** | DGANLSSARGILSLIQSSTRRAYQ |
| **8-8** | DGANLSSARGILSLIQSSTRR |
| **8-9** | DGANLSSARGILSLIQSS |
| **8-10** | DGANLSSARGILSLI |

**Supplementary Table S3.** Clinico-pathological characteristics of non-small cell lung cancer (NSCLC) patient series used in this study (n=72)\*.

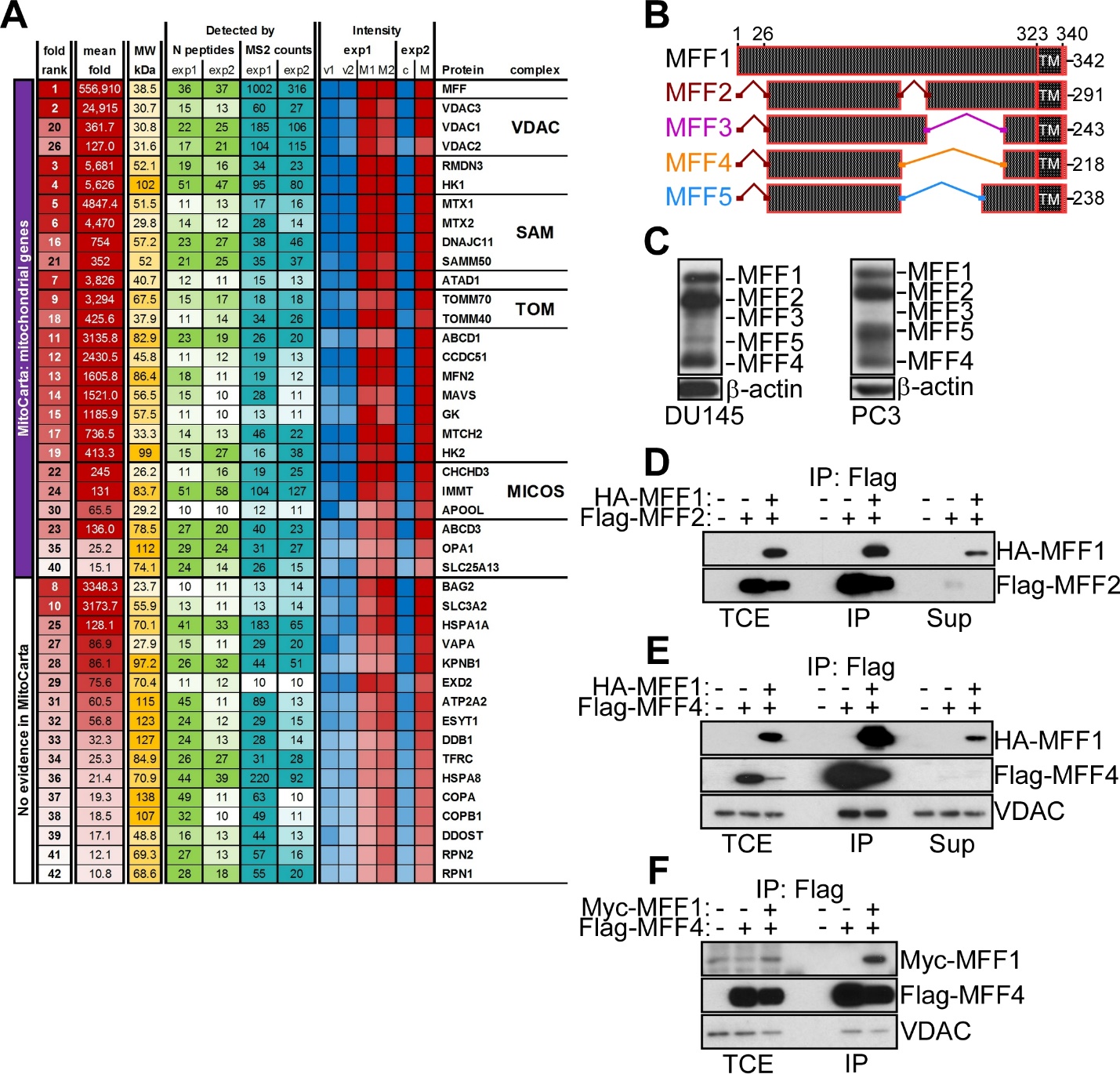
|  |  |  |
| --- | --- | --- |
|  | Feature | n |
| Histotype | Adenocarcinoma (AdCa)  Squamous Cell Carcinoma (SCC) | 57  15 |
| pN | N0  N1,2 | 45  26 |
| pT | pT1a,b  pT2a,b  pT3,4 | 24  30  18 |
| Grade | na  Grade 1  Grade 2  Grade 3 | 2  4  36  30 |
|  | ALK-R\*\* | 15 |

\*67 out of 72 samples were evaluable for MFF expression by immunohistochemistry.

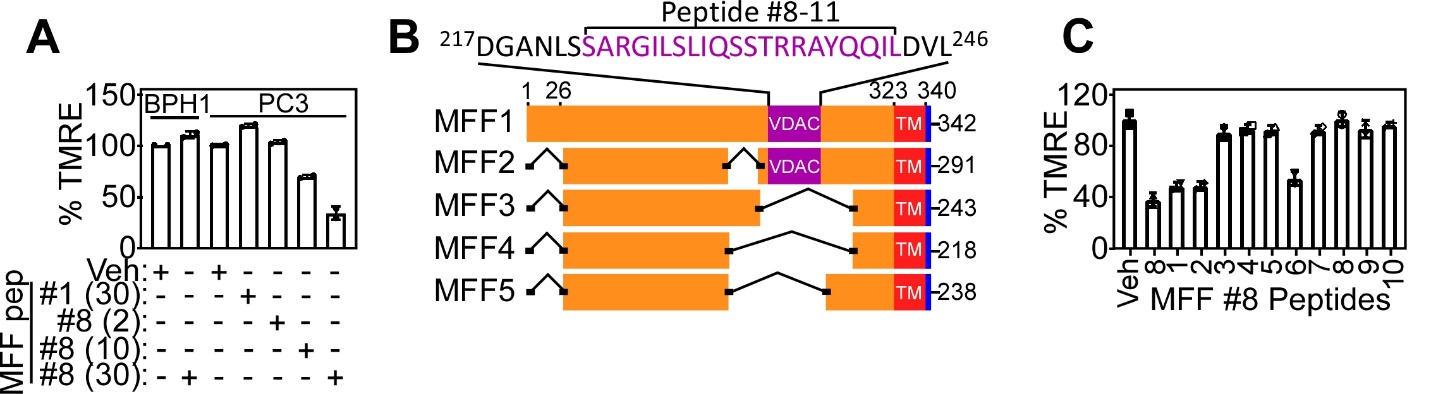
\*\* ALK-R, ALK rearrangement.

**SUPPLEMENTARY FIGURE LEGENDS**

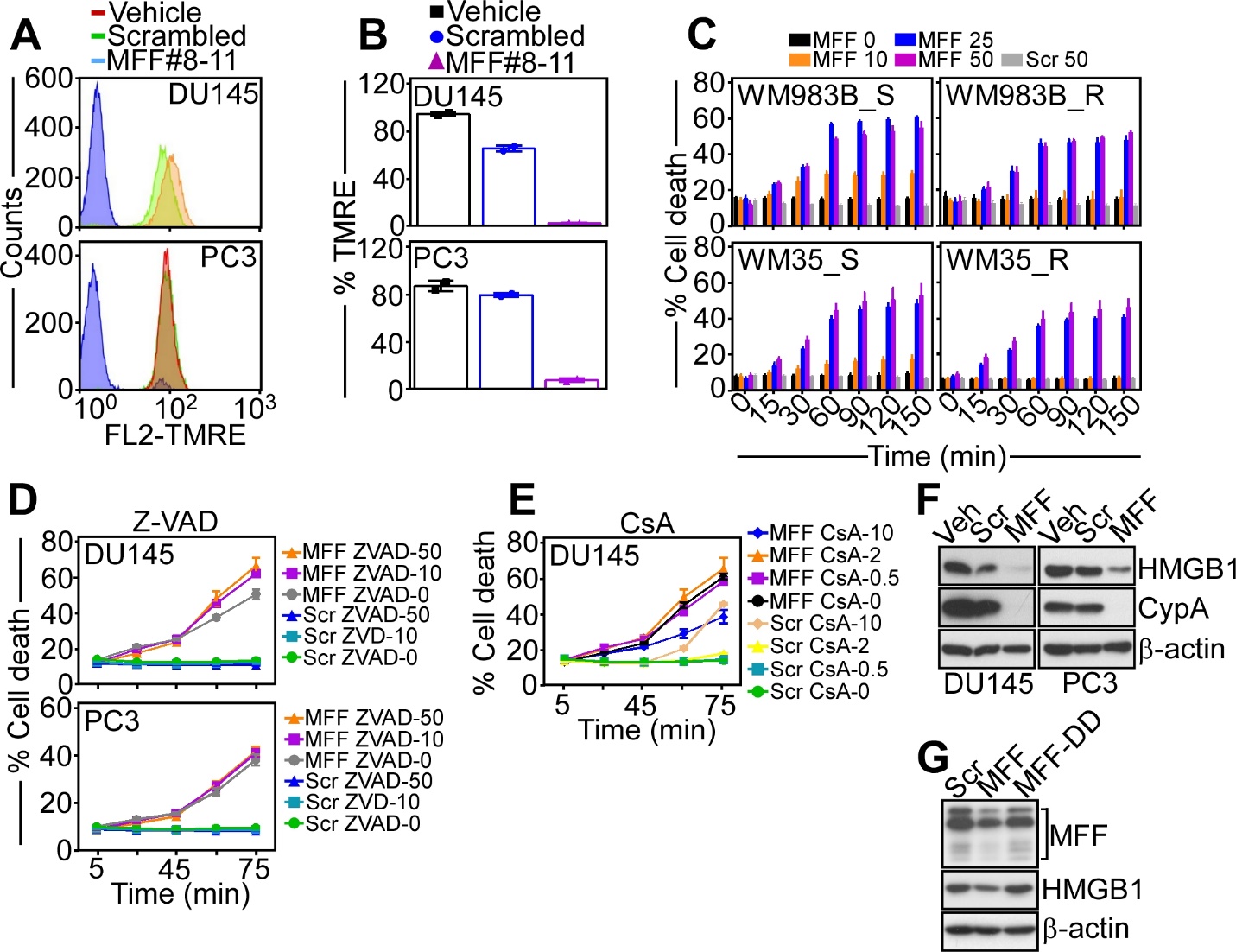
**Supplementary Fig. S1**. MFF expression in cancer. **A** and **B**, MFF expression quantified in a cohort of non-small cell lung cancer (NSLC) patients by immunohistochemistry was correlated to lymph node status (**A**) or tumor size (**B**). **C**, MFF mRNA expression and copy number in the Cancer Cell Line Encyclopedia. *Inset*, MFF mRNA expression in prostate cancer cell lines.



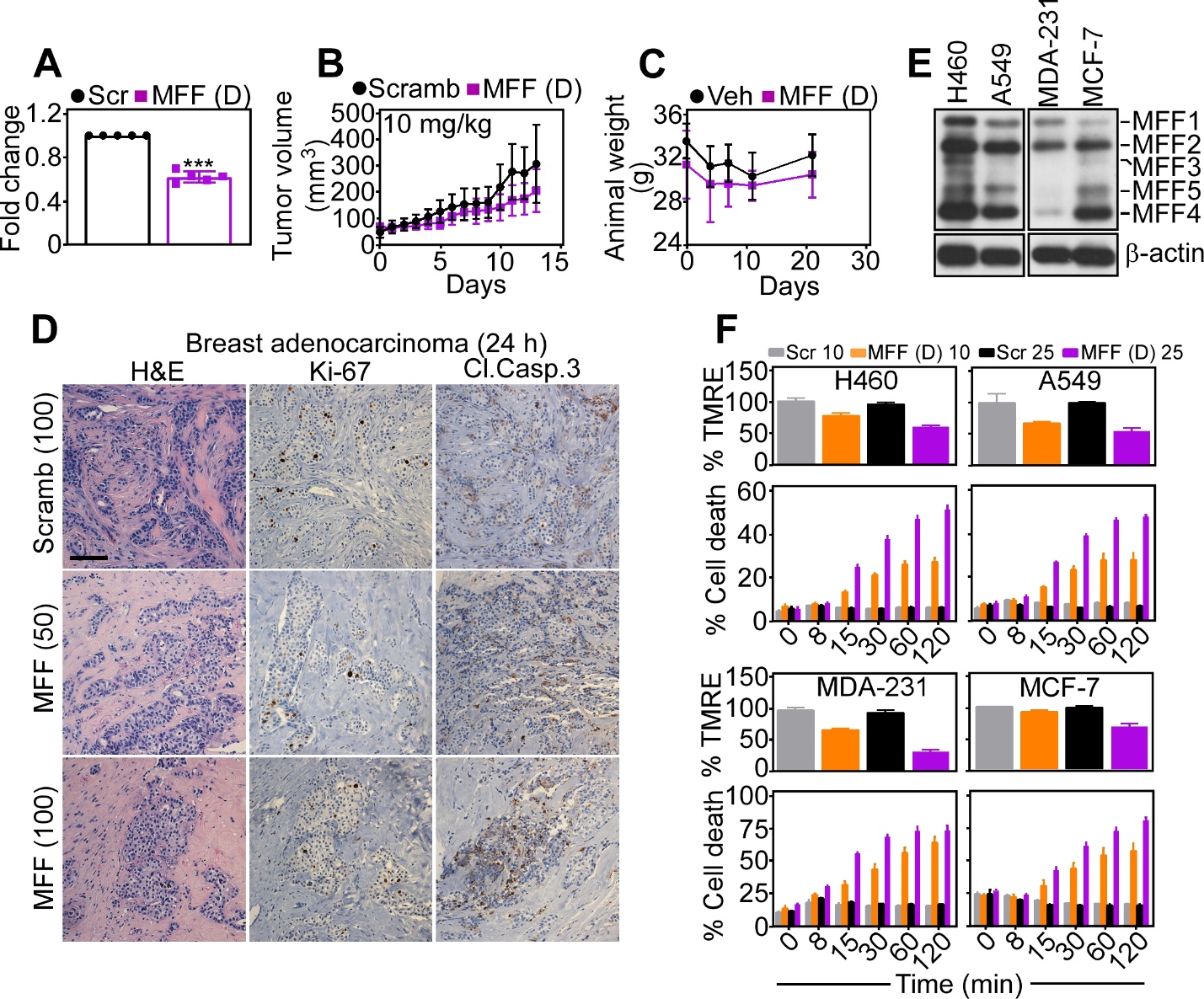
**Supplementary Fig. S2**.MFF-VDAC1 complex in cancer. **A**, Heat map of MFF-associated proteins identified by a 1D proteomics screening in PC3 cells. The mean fold, number of peptides and MS counts in two independent experiments are shown. The four main categories of MFF-associated proteins by pathway analysis (VDAC, SAM, TOM, MICOS) are shown on the right. **B**, Schematic diagram of predicted human MFF isoforms generated by alternative splicing of a single *MFF* locus. TM, transmembrane domain. The nomenclature of MFF isoforms by numbers is indicated. **C**, Total extracts from DU145 (left) or PC3 (right) cells were analyzed by Western blotting. The position of individual MFF isoforms is indicated. **D-F**, PC3 cells transfected with vector or the indicated HA- or Flag-tagged MFF cDNAs were immunoprecipitated (IP) with an antibody to Flag and analyzed by Western blotting. TCE, total cell extracts; Sup, supernatant.



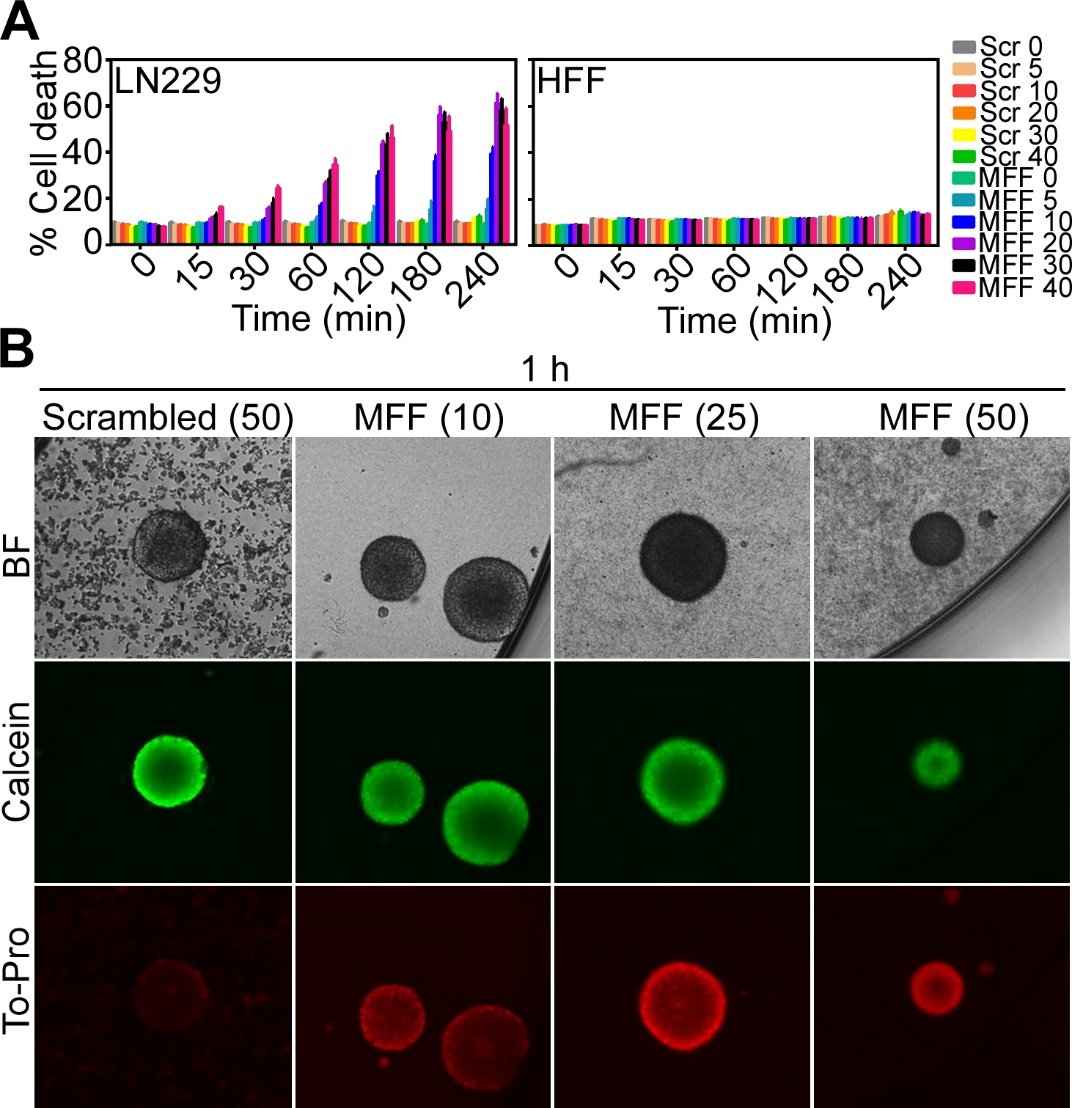
**Supplementary Fig. S3**.Peptidyl mimicry of MFF-VDAC1 complex. **A**, Mitochondrial extracts from PC3 or BPH-1 cells were incubated with MFF peptide #1 (30 M) or MFF peptide #8 (10-30 M) and analyzed for changes in mitochondrial inner membrane potential by TMRE staining and flow cytometry. Mean±SD (n=3). **B**, Schematic diagram of MFF isoforms (MFF1-MFF5) and position of a VDAC binding site in MFF1 and MFF2. The minimal MFF-VDAC1 interacting region (peptide #8-11) is indicated. TM, transmembrane domain. **C**, Mitochondrial extracts from PC3 cells were incubated with MFF peptide #8 variants as in Supplementary Table S2 and analyzed for changes in TMRE fluorescence by flow cytometry. Mean±SD (n=2).



**Supplementary Fig. S4**. Requirements of MFF regulation of tumor cell death. **A** and **B**, DU145 or PC3 cells were incubated with vehicle, cell-permeable scrambled peptide (Scrambled) or cell-permeable MFF peptide #8-11 (MFF #8-11), and TMRE staining determined by flow cytometry (**A**) was quantified (**B**). Mean±SD. **C**, Isogenic pairs of melanoma cell lines sensitive (WM983B\_S, WM35\_S) or resistant (WM983B\_R, WM35\_R) to the combination of Debrafenib plus Trametinib were incubated with increasing concentrations of the cell-permeable MFF peptide #8-11 or cell-permeable scrambled peptide (50 M) and analyzed for cell death by CellTox reactivity at the indicated time intervals. Mean±SD (n=2). **D** and **E**, DU145 or PC3 cells were incubated with cell-permeable scrambled peptide or cell-permeable MFF peptide #8-11 (0-50 M), mixed with the indicated concentrations of Z-VAD-fmk (0-50 M for 75 min, **D**) or cyclosporine A (CsA, 0-10 M for 75 min, **E**), and analyzed for cell death at the indicated time intervals. Mean±SD (n=2). (**F**) DU145 or PC3 cells were incubated with cell-permeable scrambled (Scr) or MFF peptide #8-11 and analyzed by Western blotting. Veh, vehicle. **G**, PC3 cells were incubated with cell-permeable scrambled peptide, MFF peptide #8-11 or MFF peptide #8-11 containing the double mutation R225D/R236D (DD) and analyzed by Western blotting.



**Supplementary Fig. S5**. Preclinical activity of MFF targeting. **A**, PC3 cells were treated with cell-permeable biotin-conjugated MFF (D) 8-11 peptidomimetic (MFF (D), 10 M) or cell-permeable scrambled peptide (Scr) and biotinylated mitochondrial extracts were treated with HABA (2-(4-Hydroxyphenylazo) benzoic acid)/avidin, followed by quantification of absorbance at 500 nm. Biotin accumulation in the mitochondrial samples displaces HABA from the HABA/Avidin complex, thus leading to reduction in absorbance. Mean±SD. \*\*\*, p<0.0001 by unpaired two-tailed *t* test. **B**, PC3 cells (5x106 in 50% Matrigel) were engrafted onto the flanks of immunocompromised athymic mice, and animals randomized in two groups were treated with cell-permeable scrambled peptide or MFF (D) 8-11 peptidomimetic (10 mg/kg, daily i.p.) with quantification of tumor growth at the indicated time intervals. Mean±SD (n=8-10). **C**, Tumor-bearing immunocompromised mice were treated with vehicle (Veh) or MFF (D) 8-11 peptidomimetic and animal weight was measured at the indicated time intervals. Mean±SD (n=5). **D**, Primary, patient-derived 3D organoids from cases of breast adenocarcinoma were treated with cell permeable scrambled peptide (100 M) or MFF (D) 8-11 peptidomimetic (50-100 M) for 24 h and stained for hematoxylin-eosin (H&E), Ki-67 or cleaved caspase-3 (Cl. Casp.3), by immunohistochemistry. Scale bar, 100 m. **E**, Extracts from the indicated NSCLC (A549, H460) or breast adenocarcinoma (MDA-231, MCF-7) cell lines were analyzed by Western blotting. The position of MFF isoforms is indicated. **F**, The indicated NSCLC and breast adenocarcinoma cell lines were incubated with increasing concentrations (10-25 M) of cell-permeable scrambled peptide (Scr) or MFF (D) 8-11 peptidomimetic and analyzed for mitochondrial membrane potential by TMRE staining and flow cytometry (top) or cell death by CellTox reactivity (bottom) at the indicated time intervals. Mean±SD (n=2).



**Supplementary Fig. S6**. Anti-glioma activity of MFF peptidomimetic. **A**, Glioblastoma (GBM) LN229 cells or normal HFF were treated with increasing concentrations of cell-permeable scrambled peptide or cell-permeable MFF peptide #8-11 (0-40 M) and analyzed for cell death at the indicated time intervals. Mean±SD (n=3). **B**,Primary, patient-derived human GBM neurospheres were treated with cell-permeable scrambled peptide (50 M) or the indicated increasing concentrations of MFF (D) 8-11 peptidomimetic (10-50 M) for 1 h, stained with calcein (live cells) or To-Pro (dead cells) and analyzed for fluorescence expression. FU, fluorescence units; BF, bright field.