Na⁺/K⁺-ATPase-associated FXYP3 Protein As An Intracellular Therapeutic Target in Cancer

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Abstract

Background

FXYP proteins associate closely with- and protect plasmalemmal Na^+/K^+ -ATPase against oxidative inhibition. One of them, FXYP3, is often overexpressed in cancers, including those of breast and pancreas. Down-regulation of overexpression in MCF-7 breast cancer cells with siRNA augments doxorubicin-induced cytotoxicity. Because down-regulation with siRNA is not readily translated therapeutically, we developed a peptide as an alternative for suppression of FXYP3.

Methods

A shortened peptide derivative of the wild-type (WT) FXYP3 protein, FXYP3-pep has the four cysteine residues in the WT protein replaced by serine, which eliminates the WT protein's protection against oxidative Na^+/K^+ -ATPase inhibition. We exposed human cancer cells to FXYP3-pep and measured cytotoxicity and caspase 3/7 activity with co-exposure to doxorubicin. We also measured effects of the peptide on expression glutathione-S-transferase π (GST- π), implicated in treatment resistance, and on expression of tumor suppressor p53. Selected experiments were performed with parallel FXYP3 suppression with siRNA or FXYP3-pep.

Results

Exposure of cells to FXYP3-pep displaced WT FXYP3 from Na^+/K^+ -ATPase. Exposure of MCF-7 breast or pancreatic BxPC-3 cancer cells that highly express FXYP3 to the peptide had little effect alone but combined with doxorubicin it significantly ($P < 0.05$) increased cytotoxicity. A peptide not mutated to eliminate FXYP3's protective effect of Na^+/K^+ -ATPase had no effect. FXYP3-pep did not augment doxorubicin's cytotoxicity against MDA-MB-468 breast and Panc-1 pancreatic cancer cells that have low- or no FXYP3 expression. Cellular FXYP3 expressions was reflected by expression of the $\alpha 1$ Na^+/K^+ -ATPase subunits but not by plasmalemmal Na^+/K^+ -ATPase function. Signals from fluorescently labeled FXYP3-pep were detected in a perinuclear distribution in BxPC-3 cells as reported for overexpressed FXYP3, α - and β Na^+/K^+ -ATPase subunits in cancer. Exposure to FXYP3-pep or to FXYP3 siRNA almost eliminated expression of GST- π . FXYP3-pep alone had no effect on p53 levels but significantly augmented a doxorubicin-induced increase, and, while the peptide alone had no effect on caspase 3/7 activity, it significantly augmented a doxorubicin-induced increase.

Conclusions

Overexpressed FXYP3 has intracellular roles beyond its accepted modulation of plasmalemmal Na^+/K^+ -ATPase. These roles can be countered with a membrane-permeable peptide derivative of FXYP3 that suppresses GST- π and enhances chemosensitivity of cancer cells overexpressing FXYP3.

Background

Members of the mammalian FXYP protein family, numbered 1–7, associate closely with Na⁺/K⁺-ATPase and modify its activity. They are expressed in a tissue-dependent manner [1] and in normal tissues, FXYP3 is predominantly expressed in the stomach and colon. However, as cancers develop, FXYP3 often becomes overexpressed in a variety of tissues, most commonly in cancers of prostate [2], breast [3] and pancreas [4].

Overexpression of FXYP3 has led to its use as a marker of malignancy. However, studies on cultured human MCF-7 breast cancer cells indicate that high expression may also be a marker of resistance to chemotherapy and radiotherapy. Such treatments further increase expression of FXYP3. Down-regulation of overexpression with siRNA reduces treatment resistance [5].

FXYP3 might confer treatment resistance because it protects Na⁺/K⁺-ATPase against inhibition induced by oxidative stress [6]. Such protection is potentially important therapeutically because Na⁺/K⁺-ATPase function is critical for cell survival, malignant tumors have strong antioxidant defence mechanisms and cytotoxic effects of many treatments are at least in part due to the increase in oxidative stress the treatments cause [7]. Na⁺/K⁺-ATPase is considered a potential target for cancer treatments [8]

We have examined if FXYP3 might affect proteins important in carcinogenesis and known to be activated by treatment-induced oxidative stress. We suppressed FXYP3 by exposing cells to siRNA or to a peptide derivative of FXYP3, FXYP3-pep, that displaces the wild-type (WT) protein from Na⁺/K⁺-ATPase. We examined whether suppression of FXYP3 alters expression of glutathione-S-transferase π (GST- π) that is implicated in treatment resistance [9] and the tumor suppressor p53 that has a multitude of roles in cancer [10], including in the expression of FXYP3 [11]. We also studied activation of caspase 3/7 that can be triggered by treatment-induced oxidative stress [10]. The peptide derivative FXYP3 we developed as a tool for the study is potentially a platform for novel therapeutic compounds.

Materials And Methods

Cell Cultures

Human pancreatic cancer cells BxPC-3, Panc-1, non-transformed human mammary epithelial MCF-10A cells, human breast cancer cells MCF-7 and MDA-MB-468 were obtained from ATCC (Manassas, VA). BxPC-3 cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) plus 25 mM HEPES buffer (Sigma-Aldrich). Panc-1 cells were cultured in DMEM (Life Technologies) supplemented with 10% FBS. MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Life Technologies) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (Merck Calbiochem), 0.5 μ g/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich) and 10 μ g/ml insulin (Sigma-Aldrich). MCF-7 cells were cultured in Minimum Essential Medium Eagle's (MEME) (Life Technologies) supplemented with 10% FBS, 10 μ g/ml insulin (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). MDA-MB-468 human breast cancer cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS

plus 25 mM HEPES buffer (Sigma-Aldrich) and 10 µg/ml insulin. All cells were supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C with 5% CO₂. All cells were used within 20 passages of thawing and were free of mycoplasma contamination confirmed using mycoplasma PCR Detection [12].

FXYD3 siRNA Transfection

A human siRNA against FXYD3 (consisting of pools of three to five target-specific 19-25nt siRNAs designed to knockdown gene expression, sc-60665), and the non-silencing control (control siRNA-A, sc-37007) were purchased from Santa Cruz Biotechnology. Cells were transfected using the siRNA Transfection Reagent/Medium (Santa Cruz Biotechnology) according to the manufacturer's instructions. FXYD3 mRNA expression level was quantified by real-time RT-PCR after exposure to FXYD3 siRNA as described previously [5]. FXYD3 protein abundance was measured by western blotting using GAPDH as a loading control [5].

Cell viability and apoptosis. Cell metabolic activity was assayed as described [13] by estimating reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5 carboxyanilide), using a kit (Cell Signaling Technology). The metabolic activity was used as a surrogate for viability. For each set of experimental groups, we performed 5 experiments with 4 replicates. Caspase-3-like activity is increased through a protease cascade during the early stage of apoptosis [14] and we measured activities of Caspase 3/7 (DEVDase) using the Caspase fluorogenic substrate (Calbiochem) as described [5].

Western Blot

Western blotting of cell lysates was performed as described [5]. Western blot chemiluminescence was read by a LAS-4000 image reader and quantified by densitometry using Multi Gauge 3.1 software (Fujifilm Life Science, Tokyo, Japan). Exposure times were adjusted to ensure that the variation in signal intensity was in the linear dynamic range [5]. Additional details are available in Supplementary Documentation.

FXYD3 derivatives

Exposure of cardiac myocytes to highly lipophilic recombinant WT FXYD3 protein displaces WT FXYD1 that is endogenous to cardiac myocytes. A Cys-mutated recombinant FXYD3 eliminates the function of WT FXYD1 to protect Na⁺/K⁺-ATPase function against oxidative inhibition [6]. The amino acid sequence of WT FXYD3 is shown in Fig. 1. Cys residues replaced with Ser in Cys-mutated derivative protein are indicated. Figure 1 also indicates a specific Cys residue identified by mutational studies in *Xenopus* oocytes as critical for the protective effect the WT protein has against oxidative Na⁺/K⁺-ATPase inhibition [6].

Since large cytoplasmic and extracellular domains are poorly conserved across the FXYD protein family [15] we predicted peptides with most of these domains eliminated but retaining binding regions of FXYD proteins to the Na⁺/K⁺-ATPase would have effects similar to full-length Cys-mutated FXYD3. We derived a

36 amino acid peptide analogue, FXYD3-pep, based on this prediction (Fig. 1). An analogue with the Cys residues of the WT protein retained was used in control experiments (Fig. 1) FXYD3-pep labelled with the fluorescent dye tetramethylrhodamine (TRITC) covalently bound to the extracellular N-terminal was used to study its distribution in cells exposed to it. Customized peptides were synthesized by Mimotopes Pty Ltd, Australia.

Patch Clamping

The whole cell patch-clamp technique was used to measure electrogenic Na^+/K^+ -ATPase pump current (I_p , arising from the 3:2 ratio of intracellular Na^+ pumped out of cells in exchange for extracellular K^+ pumped in). Solutions and voltage-clamp protocols were designed to minimize non-pump currents. Patch pipette solutions that perfuse the intracellular compartment after the whole-cell voltage clamp configuration is established contained (in mM): 1 NaH_2PO_4 , 5 HEPES, 5 EGTA, 2 MgATP, 86 Na^+ -glutamate, and 70 tetramethylammonium chloride. The solution was titrated to a pH of 7.2 at 22 °C using 2 M NaOH. The final concentration of Na^+ was 100 mM which causes near-maximal plasmalemmal Na^+/K^+ -ATPase activation at intracellular sites.

Cells were initially superfused with modified Tyrode's solution containing (in mM): 140 NaCl, 5.6 KCl, 2.16 CaCl_2 , 0.44 NaH_2PO_4 , 10 glucose, 1.0 MgCl_2 and 10 HEPES. The solution was titrated to a pH of 7.55 at 22 °C with NaOH. After the whole cell configuration was established, the superfusate was switched to one that was nominally Ca^{2+} -free and contained 0.2 mM CdCl_2 and 2 mM BaCl_2 . Cd^{2+} was included to block Ca^{2+} channel conductance and inhibit $\text{Na}^+/\text{Ca}^{2+}$ exchange [16]. Ba^{2+} was included to block K^+ channels. Cells were voltage clamped at 0 mV to inactivate voltage-sensitive Na^+ channels [17] and L-type Ca^{2+} channels [18, 19].

I_p was identified as the inward shift in holding current with exposure to K^+ -free extracellular solutions to eliminate Na^+/K^+ -ATPase activation at extracellular sites as described previously for measurements in cardiac myocytes [20]. We switched to the K^+ -free superfusate 2–3 minutes after the whole-cell configuration had been established. Na^+/K^+ -ATPase pump currents are small relative to other membrane currents and it is important for their accurate measurement that holding currents are stable before and after the switch from K^+ -containing to K^+ -free solutions. As for measurement of I_p in cardiac myocytes [20] we used predetermined criteria for stability of holding currents before and after changing to K^+ -free extracellular solution. I_p was normalized for cell membrane capacitance, and hence, cell size.

Fluorescent confocal microscopy

BxPC-3 cells were exposed to FXYD3-pep or to FXYD3-pep labeled with TRITC. Cells were exposed to the peptides for 2 hours, the media removed, peptide-free fresh media added, and cells then incubated for further 24 hrs. They were then fixed in 3.7% paraformaldehyde, washed and mounted on non-coated color frost slides in Fluoroshield mounting medium with DAPI (ab104139, Abcam) and examined under a laser scanning confocal microscope (Leica TCS SP5, Germany). The excitation wavelength was 543 nm, and

the emission wavelength was 572 nm. The fluorescence images were obtained using constant settings of scanning speed, pinhole diameter and voltage gain.

Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD). IC50 values for doxorubicin (Dox) in cell viability studies were calculated by GraphPad Prism. Unless otherwise indicated statistical comparisons were made with a Student's t-test or ANOVA. $P < 0.05$ was considered statistically significant.

Results

FXYD3-siRNA transfection and doxorubicin-induced cytotoxicity on BxPC-3 cells

We examined if the enhancement of doxorubicin's (Dox) cytotoxicity with siRNA-induced suppression of overexpressed FXYD3 on MCF-7 breast cancer cells [5] is reproduced in BxPC-3 pancreatic cancer cells. Cells were exposed to FXYD3 siRNA for 24 or 48 hours. Control cells were transfected with non-silencing siRNA. There are 2 splice variants of FXYD3, FXYD3a and FXYD3b. Knock-down of FXYD3a and FXYD3b mRNA (Fig. 2A) and FXYD3 protein (Fig. 2B) had similar effects to those in MCF-7 cells [5]. The Panc-1 pancreatic cancer cell line does not express detectable levels of FXYD3 and was therefore not subject to siRNA-induced FXYD3 knockdown. Effects of exposure of BxPC-3 cells to Dox alone or to Dox after FXYD3 siRNA transfection are shown in Fig. 2C. FXYD3 siRNA transfection alone had no significant effects on viability or caspase 3/7 activation, but transfection enhanced a Dox-induced decrease in viability (Fig. 2D) and caspase 3/7 activation (Fig. 2E).

FXYD3 peptide derivatives and cytotoxicity of doxorubicin

To determine if FXYD3-pep can displace WT FXYD3 from Na^+/K^+ -ATPase we exposed BxPC-3 cells to the peptide for 2 hours. Using an antibody directed against an epitope in WT FXYD3 that is absent in the shortened peptide 1 μM FXYD3-pep reduced the co-immunoprecipitation (Co-IP) of the large catalytic Na^+/K^+ -ATPase $\alpha 1$ subunit with FXYD3 by $\sim 50\%$ (Fig. 3A). The reverse Co-IP was similarly reduced (Fig. 3B).

We examined if displacement of WT FXYD3 from Na^+/K^+ -ATPase is reflected in viability of cells exposed to FXYD3-pep with and without co-exposure to Dox for 48 hours. Culture medium that contained FXYD3-pep and Dox was replaced with fresh medium every 24 hours.

FXYD3-pep augmented effects of Dox on the pancreatic BxPC-3 cells that highly express FXYD3. There was a shift in IC50 for Dox from $\sim 1.6 \mu\text{M}$ for cells not exposed to FXYD3-pep to $\sim 0.4 \mu\text{M}$ and $\sim 0.3 \mu\text{M}$ with exposure to 1 μM and 2 μM FXYD3-pep (Fig. 4A). FXYD3-pep did not augment the effect of Dox on pancreatic Panc-1 cells that do not express FXYD3 (Fig. 4B). FXYD3-pep augmented effects of Dox on the MCF-7 cell line with a shift in IC50 from $\sim 3.4 \mu\text{M}$ without exposure to it to $\sim 0.5 \mu\text{M}$ with exposure to

2 μ M FXYD3-pep (Fig. 4C). FXYD3-pep did not augment effects of Dox on the breast cancer cell line MDA-MB-468 that expresses FXYD3 at a much lower level than the MCF-7 cells. (Fig. 4D). The peptide alone at 1 or 2 μ M had no effect on viability of any of the pancreatic or breast cancer cell lines.

We also examined the effect of the peptide, FXYD3-pepC (Fig. 1C), identical to FXYD3-pep except that it retains the Cys residues of WT FXYD3 that were mutated to Ser in FXYD3-pep. In contrast to co-exposure of BxPC-3 cells to FXYD3-pep and Dox (Fig. 4A), FXYD3-pepC did not augment Dox-induced cytotoxicity (Fig. 4E). It also did not augment Dox-induced cytotoxicity of Panc-1 cells (Fig. 4F). These results implicate mutation of the Cys residues rather than elimination of cytosolic- and extracellular parts of WT FXYD3 in FXYD3-pep's augmentation of cytotoxicity.

Cellular target for FXYD3-pep

Plasmalemmal Na^+/K^+ -ATPase is critical for cell survival and since WT FXYD3 protects Na^+/K^+ -ATPase against inhibition induced by oxidative stress [6] we examined if inhibition of Na^+/K^+ -ATPase augments Dox-induced cytotoxicity. We exposed MCF-7 cells to ouabain that binds to plasma membrane Na^+/K^+ -ATPase at extracellular sites and inhibits its activity. Exposure of cells to ouabain for 48 hours, with or without co-exposure to Dox reduced cell viability in a concentration-dependent manner but did not augment effects of Dox (Fig. 5A).

Levels of the $\alpha 1$ Na^+/K^+ -ATPase subunit, normalized to levels in the non-transformed human breast cell line MCF-10A, were much higher in the BxPC-3 and MCF-7 cells that were sensitized to effects of Dox by FXYD3-pep than in the MDA-MB-468 and Panc-1 cells that were not sensitized. Overexpression of FXYD3 broadly followed the same pattern as $\alpha 1$ Na^+/K^+ ATPase subunit expression (Fig. 5B). We did not detect WT FXYD3 protein or FXYD3 mRNA for Panc-1 (not shown) as others also reported [4].

We examined if the ~ 15 -fold higher signal for $\alpha 1$ Na^+/K^+ -ATPase subunits in MCF-7 than MDA-MB-468 cells (Fig. 5B) is reflected by a difference in the cells' functional electrogenic plasmalemmal Na^+/K^+ -ATPase current, I_p . I_p was measured under conditions causing near-maximal Na^+/K^+ -ATPase turnover and hence reflecting plasmalemmal Na^+/K^+ -ATPase abundance. I_p was similar for MCF-7 and MDA-MB-468 cells (Fig. 5C).

The similar plasmalemmal near-maximal Na^+/K^+ -ATPase pump capacity of MCF-7 and MDA-MB-468 cells despite their difference in $\alpha 1$ Na^+/K^+ -ATPase subunit expression implicate the existence a cellular pool of Na^+/K^+ -ATPase not residing in the plasmalemma in cells sensitized to Dox by FXYD3-pep.

To examine the cellular distribution of FXYD3-pep labeled with the fluorescent dye TRITC. we used pancreatic BxPC-3 cells because they exhibited the strongest signal for expression of $\alpha 1$ Na^+/K^+ -ATPase subunits (Fig. 5B) that FXYD3-pep is expected to bind to. TRITC fluorescence was detected in the cytosol, particularly in the perinuclear region (Fig. 5D). Fluorescence was not detected in cells exposed to unlabeled FXYD3-pep (data not shown). Fluorescence reflecting binding to plasmalemmal Na^+/K^+ -ATPase

was not selectively detected. However, a weak signal from such fluorescence is likely to be undetectable with overlap of signals in multi-layered cultured cells.

Cellular response to FXYD3-pep

Overexpression of GST π often confers treatment resistance, at least in part by protecting the cancer cells against treatment-induced oxidative stress [9]. Exposure of BxPC-3 cells to 1 μ M FXYD3-pep for 24 hours reduced GST π expression by $\sim$ 85% (Fig. 6A). A reduction consistent with this occurred with exposure to the full-length recombinant Cys-mutated FXYD3 protein (Fig. 6B). Data pooled from Fig. 6A and Fig. 6B with exposure of cells to FXYD3-pep or the full-length Cys-mutated protein is quantitated in Fig. 6C. Exposure to FXYD3 siRNA also appeared to reduce GST π expression and it appeared to block a Dox-induced increase in GST π (Fig. 6D). We did not examine effects of suppressing FXYD3 on GST π in MCF-7 cells because they are reported to express GST π at very low levels [21]. We did not detect it in the MCF-7 cells here (data not shown).

To examine if GST π directly facilitates glutathionylation of the β 1 Na⁺/K⁺ ATPase subunit we incubated Na⁺/K⁺ ATPase-enriched membrane fragments with GST π . A clear signal for GST π -induced β 1 subunit glutathionylation was detected in 2 experiments (Supplementary Fig S1).

GST π has to bind to its target protein to facilitate glutathionylation [21] and we examined if oxidative stress induced by a physiological stimulus increases association between the β 1 Na⁺/K⁺ ATPase subunit and GST π . Exposure of isolated cardiac myocytes to angiotensin II to activate NADPH oxidase [22] appeared to increase co-immunoprecipitation of GST π with the β 1 subunit in 4 experiments (Supplementary Fig S2).

The tumor suppressor p53 promotes both pro- and anti-apoptotic signaling in response to treatment-induced DNA damage and oxidative stress [23]. We examined abundance of p53 after exposure of cells to FXYD3-pep. Exposure of MCF-7 and BxPC-3 cells to 1 μ M FXYD3-pep for 24 hours had no effect in either cell line. We also exposed BxPC-3 cells to 0.5 μ M Dox and MCF-7 cells to 2.5 μ M Dox for 48 hours, with or without exposure to 1 μ M FXYD3-pep. When used, FXYD3-pep was added for 24 hours before cells were also exposed to Dox. The different concentrations of Dox were chosen to reflect differences in sensitivity to cytotoxicity of Dox with exposure to 1 μ M FXYD3-pep (Fig. 4). MCF-7 cells expressed low levels of p53 at baseline. Exposure to Dox alone increased expression of p53 for both cell lines and this was augmented with exposure to FXYD3-pep (Fig. 6E).

GST π inhibits apoptotic signalling induced by oxidative stress and the reduced expression of GST π FXYD3-pep causes (Fig. 6A -C) might augment apoptotic signalling. We examined effects of FXYD3-pep on caspase 3/7 activation with and without the oxidative stress caused by Dox exposure. Exposure to 1 μ M FXYD3-pep for 24 hours alone had no effect on Caspase 3/7 activity in cytosolic extracts of MCF-7 and BxPC-3 cells but pre-exposure of the cells to FXYD3-pep for 24 hours followed by 48 hours co-exposure to FXYD3-pep and 0.5 μ M Dox for BxPC-3 cells or 2.5 μ M Dox for MCF-7 cells augmented a Dox-

induced increase in Caspase 3/7 activity (Fig. 6F). The net effect of FXYD3-pep is to augment Dox-induced apoptotic signaling.

In vivo uptake of FXYD3-pep

Displacement of WT FXYD proteins from Na^+/K^+ -ATPase in non-cancer cells might cause toxicity that would preclude use of a therapeutic peptide developed from FXYD-pep. In preliminary studies we examined if acute adverse effects were detectable. Fluorescence monitored in mice after intraperitoneal injection TRITC-labeled FXYD-pep indicated systemic uptake, independently confirmed by fluorescence microscopy of the myocardium (Supplementary Fig S3). There were no apparent adverse effects of the peptide.

Discussion

Expression of the large Na^+/K^+ -ATPase α subunits was much higher for MCF-7 breast cancer cells than MDA-MB-468 cells but electrogenic plasmalemmal Na^+/K^+ -ATPase currents were similar suggesting the presence of a pool of Na^+/K^+ -ATPase in MCF-7 cells that does not contribute to plasmalemmal Na^+/K^+ -transport. In agreement with this Na^+/K^+ -ATPase α subunits are strongly expressed in the intracellular compartment of MCF-7 cells [3]. Intracellular distribution of overexpressed α subunits also occurs in cancer cells of other tissues [24, 25]. We are not aware of reports of parallel intracellular expression of α/β subunits to implicate expression of functional Na^+/K^+ -ATPase. However, ouabain-sensitive $^{86}\text{Rb}^+$ uptake mediated by Na^+/K^+ -ATPase in the plasmalemma does not reflect variable $\beta 1$ subunit expression in cancer cells [26]. This implicates the existence of non-plasmalemmal pools of $\beta 1$ subunits and is in turn consistent with non-plasmalemmal intracellular pools of intact α/β complexes.

Intracellular TRITC-labeled FXYD3-pep fluorescence in BxPC-3 reflects the distribution of overexpressed FXYD3 protein in resected sections of pancreatic- [4] and breast cancers [3] and the strongest fluorescence in the perinuclear region is similar to the distribution of overexpressed FXYD3 and Na^+/K^+ -ATPase α subunits in MCF-7 cells [3]. Binding of TRITC-labeled FXYD3-pep to overexpressed α subunits in BxPC-3 cells, if in the same distribution, would account for the pattern of TRITC fluorescence here.

Fluorescence from a 2-hour exposure to TRITC-labeled FXYD3-pep was retained 24 hours after the peptide was washed off BxPC-3 cells and since the peptide is membrane-permeable it was expected to exit cells unless bound. Consistent with this, in MCF-7 cells there is no WT FXYD3 protein that might have been freely diffusible in the cytosolic fraction while the membranous fraction contains FXYD3 in easily detectable amounts [3].

Experimentally induced synthesis of FXYD3 in non-cancer Chinese Hamster Ovary (CHO) cells suggest how enhanced synthesis of FXYD3 might drive accumulation of Na^+/K^+ -ATPase in intracellular membranes. FXYD3 expressed in the CHO cells was distributed in the nuclear envelope and the endoplasmic reticulum (ER) but not in the plasmalemma [27]. The FXYD3 should facilitate local

assembly of the α - with β Na^+/K^+ -ATPase subunits because FXYD proteins have bonds to both [28]. Increased α/β subunit co-immunoprecipitation with induced expression of FXYD1 in *Xenopus* oocytes or exposure of cardiac myocytes to recombinant WT FXYD3 [6] show how abundance of FXYD proteins facilitate Na^+/K^+ -ATPase assembly. Epitopes of FXYD3 with bonds to Na^+/K^+ -ATPase are preserved in FXYD3-pep and these should retain the peptide intracellularly, bound to overexpressed Na^+/K^+ -ATPase.

While functional α/β Na^+/K^+ -ATPase is often taken to exist only in the plasmalemma of non-cancer cells it is also found in the nuclear envelope where in human embryonic kidney 293 cells it maintains a transmembrane concentration gradient for Na^+ . This allows $\text{Na}^+/\text{Ca}^{2+}$ exchange-mediated Ca^{2+} efflux from the nucleoplasm into the nucleoplasmic reticulum [29]. Ca^{2+} overload of the nucleoplasm in a variety of cells directly affects chromatin organization and activates cell death signaling and apoptosis [30].

A Na^+/K^+ -ATPase inhibitor might cause Ca^{2+} overload of the nucleoplasm but here the selective inhibitor ouabain did not augment Dox's cytotoxicity. However, ouabain is poorly membrane-permeable and might not traverse the outer membrane of the nuclear envelope to access Na^+/K^+ -ATPase in the inner membrane. In support of this it had no effect on ATP-activated Na^+ efflux or $\text{Na}^+/\text{Ca}^{2+}$ exchange-coupled Ca^{2+} efflux in cell nuclei that in contrast was almost eliminated by the membrane-permeable Na^+/K^+ -ATPase inhibitor strophanthidin [29].

Membrane permeability of FXYD3-pep allows it to reach Na^+/K^+ -ATPase in intracellular membranes and displacement of WT FXYD3 is expected to amplify $\beta 1$ subunit glutathionylation and inhibition of Na^+/K^+ -ATPase with Dox-induced oxidative stress. Such inhibition should alter Ca^{2+} levels in intracellular compartments and, in the ER, an increase in these levels is implicated in Dox-induced apoptosis [31]. However, to our knowledge, functional Na^+/K^+ -ATPase has not been found in ER membranes of cancer- or non-cancer cells.

Co-localization of FXYD3 with Na^+/K^+ -ATPase α subunits in MCF-7 cells in an intracellular distribution [3] similar to that for co-localization of FXYD3 with the tyrosine kinase Src [32] implicates a mechanism independent of Na^+/K^+ -ATPase inhibition in FXYD3-pep's augmentation of cytotoxicity. Src's SH domain interacts with FXYD3, the same domain that also binds Src to the second cytosolic domain of the Na^+/K^+ -ATPase $\alpha 1$ subunit [33]. The Src family of tyrosine kinases is redox regulated [34] and the reactive Cys in FXYD3 (Fig. 1) might optimize redox balance. FXYD3 expression is promoted by the stem cell-related transcription factor SOX9 and FXYD3 in turn is critical for confining SOX9 to the nucleus. Knock down of FXYD3 with shRNA reduces the stem cell population and markedly impairs growth of MCF-7 xenograft tumors in mice. In this scheme of a SOX9/FXYD3/Src axis [32], Na^+/K^+ -ATPase α subunits serve to anchor FXYD3 intracellularly rather than that of a functional target for FXYD3-pep.

FXYD3-pep, full-length cysteine-mutated FXYD3 or FXYD3 siRNA almost eliminated expression of GST π in BxPC-3 cells. GST π is expressed in the cytosol, nucleus [35] and ER [36] and is widely implicated in

drug resistance of cancers. The resistance has been attributed to GST π aiding in the export of toxins from cells, including export of chemotherapeutics. [21]. Alternative mechanisms have been proposed, including a role for GST π in inducing glutathionylation of proteins [21].

While glutathionylation of the β 1 subunit inhibits Na⁺/K⁺-ATPase [37], glutathionylation can also protect protein cysteine residues against irreversible oxidation, preserving proteins in a reversible functionally silent state. GST π promotes such protection [38]. Binding of GSH to GST π lowers GSH's cysteine thiol pKa, forming a nucleophilic thiolate anion that GST π can deliver to cysteine residues in hydrophobic domains. The susceptible cysteine residue in the β 1 Na⁺/K⁺-ATPase subunit is in such a domain [37] and results here suggest GST π can catalyze its glutathionylation.

β subunits are N-terminal glycosylated membrane proteins and MDA-MB-468 and Panc-1 cells share the p53 R273H mutation that promotes folding of such proteins in the ER [39]. The normal folding of β subunits is important for its assembly with α subunits. However, it is not apparent if this somehow relates to the low- or absent expression of FXYD3 in these cells, contrasting FXYD3 expression in the MCF-7 and BxPC-3 cells.

We are not aware of direct interdependence of FXYD3 and GST π that might account for FXYD3-pep's down-regulation of GST π but note synthesis of both FXYD3 [11] and GST π [40] depend on p53. Exposure to FXYD3-pep alone had no effect on p53 abundance, yet it markedly decreased GST π expression. Interdependence of FXYD3 and GST π via common p53-dependent synthesis cannot be implicated from our data.

Conclusions

We have for the first time established a link between overexpression of FXYD3 and GST π . GST π is a recognized target for treatment resistance and many GST π inhibitors are under development [41]. Many peptide drugs are already in clinical use or under development [42] and we propose the membrane-permeable FXYD-pep is a promising platform for development into a drug that selectively sensitizes cancers overexpressing cytosolic FXYD3 to chemotherapeutics, in particular when combined with a GST π inhibitor. Targeting FXYD3 might also address the treatment resistance that cancer stem cells confer, at least in some breast cancers [32].

Declarations

Ethics approval and consent to participate

Ethics approval was not required as studies were conducted on cell lines only.

Consent for publication

Not applicable

Availability of data and materials

The datasets collected during the current study are available from the corresponding author on reasonable request.

Competing interests

HHR is a named inventor of a patent owned by Northern Sydney Local Health District on the use of FXYD3 derivatives in cancer treatment. No commercial arrangements are entered into.

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Authors' contributions

CCL acquired, analysed and interpreted data. YJK, RT, AG, AWH and EJH acquired experimental data. EJH also participated in drafting the manuscript. FC, RCB and HHR made substantial contributions to the conception and design of experiments. HHR also interpreted data and drafted the manuscript. All authors reviewed the final manuscript.

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References

1. Sweadner KJ, Rael E. The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. *Genomics*. 2000;68(1):41–56. doi:10.1006/geno.2000.6274.
2. Grzmil M, Voigt S, Thelen P, Hemmerlein B, Helmke K, Burfeind P. Up-regulated expression of the MAT-8 gene in prostate cancer and its siRNA-mediated inhibition of expression induces a decrease in proliferation of human prostate carcinoma cells. *Int J Oncol*. 2004;24(1):97–105.

3. Yamamoto H, Okumura K, Toshima S, Mukaisho K, Sugihara H, Hattori T, Kato M, Asano S. FXYD3 protein involved in tumor cell proliferation is overproduced in human breast cancer tissues. *Biol Pharm Bull.* 2009;32(7):1148–54. doi:10.1248/bpb.32.1148.
4. Kayed H, Kleeff J, Kolb A, Ketterer K, Keleg S, Felix K, Giese T, Penzel R, Zentgraf H, Buchler MW, Korc M, Friess H. FXYD3 is overexpressed in pancreatic ductal adenocarcinoma and influences pancreatic cancer cell growth. *Int J Cancer.* 2006;118(1):43–54. doi:10.1002/ijc.21257.
5. Liu CC, Teh R, Mozar CA, Baxter RC, Rasmussen HH. Silencing overexpression of FXYD3 protein in breast cancer cells amplifies effects of doxorubicin and gamma-radiation on Na⁺/K⁺-ATPase and cell survival. *Breast Cancer Res Treat.* 2016;155(2):203–13. doi:10.1007/s10549-015-3667-x.
6. Bibert S, Liu CC, Figtree GA, Garcia A, Hamilton EJ, Marassi FM, Sweadner KJ, Cornelius F, Geering K, Rasmussen HH. FXYD proteins reverse inhibition of the Na⁺-K⁺ pump mediated by glutathionylation of its beta1 subunit. *J Biol Chem.* 2011;286(21):18562–72. doi:10.1074/jbc.M110.184101.
7. Pani G, Giannoni E, Galeotti T, Chiarugi P. Redox-based escape mechanism from death: the cancer lesson. *Antioxid Redox Signal.* 2009;11(11):2791–806. doi:10.1089/ars.2009.2739.
8. Mijatovic T, Dufrasne F, Kiss R. Cardiotonic steroids-mediated targeting of the Na⁺/K⁺-ATPase to combat chemoresistant cancers. *Curr Med Chem.* 2012;19(5):627–46. doi:10.2174/092986712798992075.
9. Singh S. Cytoprotective and regulatory functions of glutathione S-transferases in cancer cell proliferation and cell death. *Cancer Chemother Pharmacol.* 2015;75(1):1–15. doi:10.1007/s00280-014-2566-x.
10. Mirzayans R, Andrais B, Kumar P, Murray D. (2017) Significance of Wild-Type p53 Signaling in Suppressing Apoptosis in Response to Chemical Genotoxic Agents: Impact on Chemotherapy Outcome. *Int J Mol Sci* 18 (5). doi:10.3390/ijms18050928.
11. Maxwell PJ, Longley DB, Latif T, Boyer J, Allen W, Lynch M, McDermott U, Harkin DP, Allegra CJ, Johnston PG. Identification of 5-fluorouracil-inducible target genes using cDNA microarray profiling. *Cancer Res.* 2003;63(15):4602–6.
12. Uphoff CC, Drexler HG. Detecting mycoplasma contamination in cell cultures by polymerase chain reaction. *Methods Mol Biol.* 2011;731:93–103. doi:10.1007/978-1-61779-080-5_8.
13. Saxena NK, Vertino PM, Anania FA, Sharma D. leptin-induced growth stimulation of breast cancer cells involves recruitment of histone acetyltransferases and mediator complex to CYCLIN D1 promoter via activation of Stat3. *J Biol Chem.* 2007;282(18):13316–25. doi:10.1074/jbc.M609798200.
14. Nicholson DW, Thornberry NA. Caspases: killer proteases. *Trends Biochem Sci.* 1997;22(8):299–306. doi:10.1016/s0968-0004(97)01085-2.
15. Cornelius F, Mahmmoud YA. Functional modulation of the sodium pump: the regulatory proteins "Fixit". *News Physiol Sci.* 2003;18:119–24.

16. Gadsby DC, Nakao M. Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J Gen Physiol.* 1989;94(3):511–37. doi:10.1085/jgp.94.3.511.
17. Follmer CH, ten Eick RE, Yeh JZ. Sodium current kinetics in cat atrial myocytes. *J Physiol.* 1987;384:169–97. doi:10.1113/jphysiol.1987.sp016449.
18. Bean BP. Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *J Gen Physiol.* 1985;86(1):1–30. doi:10.1085/jgp.86.1.1.
19. Krafte DS, Kass RS. Hydrogen ion modulation of Ca channel current in cardiac ventricular cells. Evidence for multiple mechanisms. *J Gen Physiol.* 1988;91(5):641–57. doi:10.1085/jgp.91.5.641.
20. Garcia A, Liu CC, Cornelius F, Clarke RJ, Rasmussen HH. Glutathionylation-Dependence of Na⁺-K⁺-Pump Currents Can Mimic Reduced Subsarcolemmal Na(+) Diffusion. *Biophys J.* 2016;110(5):1099–109. doi:10.1016/j.bpj.2016.01.014.
21. Tew KD, Manevich Y, Grek C, Xiong Y, Uys J, Townsend DM. The role of glutathione S-transferase P in signaling pathways and S-glutathionylation in cancer. *Free Radic Biol Med.* 2011;51(2):299–313. doi:10.1016/j.freeradbiomed.2011.04.013.
22. White CN, Figtree GA, Liu CC, Garcia A, Hamilton EJ, Chia KK, Rasmussen HH. Angiotensin II inhibits the Na⁺-K⁺ pump via PKC-dependent activation of NADPH oxidase. *Am J Physiol Cell Physiol.* 2009;296(4):C693–700. doi:10.1152/ajpcell.00648.2008.
23. Hientz K, Mohr A, Bhakta-Guha D, Efferth T. The role of p53 in cancer drug resistance and targeted chemotherapy. *Oncotarget.* 2017;8(5):8921–46. doi:10.18632/oncotarget.13475.
24. Baker Bechmann M, Rotoli D, Morales M, Maeso Mdel C, Garcia Mdel P, Avila J, Mobasher A, Martin-Vasallo P. Na,K-ATPase Isozymes in Colorectal Cancer and Liver Metastases. *Front Physiol.* 2016;7:9. doi:10.3389/fphys.2016.00009.
25. Yang P, Cartwright C, Efuet E, Hamilton SR, Wistuba II, Menter D, Addington C, Shureiqi I, Newman RA. Cellular location and expression of Na⁺, K⁺-ATPase alpha subunits affect the anti-proliferative activity of oleandrin. *Mol Carcinog.* 2014;53(4):253–63. doi:10.1002/mc.21968.
26. Espineda CE, Chang JH, Twiss J, Rajasekaran SA, Rajasekaran AK. Repression of Na,K-ATPase beta1-subunit by the transcription factor snail in carcinoma. *Mol Biol Cell.* 2004;15(3):1364–73. doi:10.1091/mbc.E03-09-0646.
27. Arimochi J, Kobayashi A, Maeda M. Stable expression and visualization of Mat-8 (FXD-3) tagged with a fluorescent protein in Chinese hamster ovary (CHO)-K1 cells. *Biotechnol Lett.* 2005;27(14):1017–24. doi:10.1007/s10529-005-7870-4.
28. Shinoda T, Ogawa H, Cornelius F, Toyoshima C. Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature.* 2009;459(7245):446–50. doi:10.1038/nature07939.
29. Galva C, Artigas P, Gatto C. Nuclear Na⁺/K⁺-ATPase plays an active role in nucleoplasmic Ca²⁺ homeostasis. *J Cell Sci.* 2012;125(Pt 24):6137–47. doi:10.1242/jcs.114959.
30. Zhivotovsky B, Orrenius S. Calcium and cell death mechanisms: a perspective from the cell death community. *Cell Calcium.* 2011;50(3):211–21. doi:10.1016/j.ceca.2011.03.003.

31. Giorgi C, Bonora M, Sorrentino G, Missiroli S, Poletti F, Suski JM, Galindo Ramirez F, Rizzuto R, Di Virgilio F, Zito E, Pandolfi PP, Wieckowski MR, Mammano F, Del Sal G, Pinton P. p53 at the endoplasmic reticulum regulates apoptosis in a Ca^{2+} -dependent manner. *Proc Natl Acad Sci U S A*. 2015;112(6):1779–84. doi:10.1073/pnas.1410723112.
32. Xue Y, Lai L, Lian W, Tu X, Zhou J, Dong P, Su D, Wang X, Cao X, Chen Y, Wang Q. SOX9/FXYD3/Src Axis Is Critical for ER(+) Breast Cancer Stem Cell Function. *Mol Cancer Res*. 2019;17(1):238–49. doi:10.1158/1541-7786.MCR-18-0610.
33. Banerjee M, Duan Q, Xie Z. SH2 Ligand-Like Effects of Second Cytosolic Domain of Na/K-ATPase alpha1 Subunit on Src Kinase. *PLoS One*. 2015;10(11):e0142119. doi:10.1371/journal.pone.0142119.
34. Dustin CM, Heppner DE, Lin MJ, van der Vliet A. Redox regulation of tyrosine kinase signaling: More than meet the eye. *J Biochem*. 2019. doi:10.1093/jb/mvz085.
35. Go YM, Jones DP. Redox control systems in the nucleus: mechanisms and functions. *Antioxid Redox Signal*. 2010;13(4):489–509. doi:10.1089/ars.2009.3021.
36. Ye ZW, Zhang J, Ancrum T, Manevich Y, Townsend DM, Tew KD. Glutathione S-Transferase P-Mediated Protein S-Glutathionylation of Resident Endoplasmic Reticulum Proteins Influences Sensitivity to Drug-Induced Unfolded Protein Response. *Antioxid Redox Signal*. 2017;26(6):247–61. doi:10.1089/ars.2015.6486.
37. Figtree GA, Liu CC, Bibert S, Hamilton EJ, Garcia A, White CN, Chia KKM, Cornelius F, Geering K, Rasmussen HH. Reversible oxidative modification: a key mechanism of Na^+ - K^+ pump regulation. *Circ Res*. 2009;105(2):185–93.
38. Popov D. Protein S-glutathionylation: from current basics to targeted modifications. *Arch Physiol Biochem*. 2014;120(4):123–30. doi:10.3109/13813455.2014.944544.
39. Vogiatzi F, Brandt DT, Schneikert J, Fuchs J, Grikscheit K, Wanzel M, Pavlakis E, Charles JP, Timofeev O, Nist A, Mernberger M, Kantelhardt EJ, Siebolts U, Bartel F, Jacob R, Rath A, Moll R, Grosse R, Stiewe T. Mutant p53 promotes tumor progression and metastasis by the endoplasmic reticulum UDPase ENTPD5. *Proc Natl Acad Sci U S A*. 2016;113(52):E8433–42. doi:10.1073/pnas.1612711114.
40. Lo HW, Stephenson L, Cao X, Milas M, Pollock R, Ali-Osman F. Identification and functional characterization of the human glutathione S-transferase P1 gene as a novel transcriptional target of the p53 tumor suppressor gene. *Mol Cancer Res*. 2008;6(5):843–50. doi:10.1158/1541-7786.MCR-07-2105.
41. Allocati N, Masulli M, Di Ilio C, Federici L. Glutathione transferases: substrates, inhibitors and pro-drugs in cancer and neurodegenerative diseases. *Oncogenesis*. 2018;7(1):8. doi:10.1038/s41389-017-0025-3.
42. Lau JL, Dunn MK. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Bioorg Med Chem*. 2018;26(10):2700–7. doi:10.1016/j.bmc.2017.06.052.

Figures

Figure 1

Amino acid sequences of FXYD3 and its derivatives. A. Wild-type (WT) FXYD3. Cys residues mutated to Ser in full-length recombinant protein and the FXYD motif in the extracellular domain are shown in bold and are underlined. The Cys residue critical for the protective effect the WT protein has against oxidative Na⁺/K⁺-ATPase inhibition is indicated by *. B. FXYD3-pep. Ser residues that displaced Cys residues in the corresponding sites in WT FXYD3 are indicated. C. Peptide analogue with Cys residues of WT FXYD3 retained.

Figure 2

Effects of FXYD3-siRNA transfection of BxPC-3 cells. A. Expression of FXYD3a and FXYD3b mRNA after exposure of cells to FXYD3 siRNA for 24 or 48 hours. N = 4. B. Expression of FXYD3 protein in cells after 48 hours exposure to FXYD3 siRNA. N = 4. C. FXYD3 siRNA transfection and effect of Dox on FXYD3 expression. Cells were exposed to 1 μM Dox or Dox-free culture medium for 48 hours after transfection as indicated (N = 3). D. FXYD3 siRNA transfection and effect of Dox on cell viability and. Cells were exposed

to Dox or Dox-free culture medium as for panel C (N = 5). E. FXYD3 siRNA transfection and effect of Dox on Caspase-3/7 activity. Cells were exposed to Dox or Dox-free culture medium as for panels C and D (N = 5).

Figure 3

Displacement of FXYD3 from $\alpha 1$ Na⁺/K⁺-ATPase subunit with exposure to FXYD3-pep. A. Immunoblot (IB) of $\alpha 1$ Na⁺/K⁺-ATPase subunit with WT FXYD3 immunoprecipitate in lysate of BxPC-3 with and without exposure of the cells to 1 μ M FXYD3-pep (pep) for 2 hours before lysis. B. Immunoblot of WT FXYD3 with the $\alpha 1$ subunit immunoprecipitate in the lysate from the cells. C: control; TL: total lysate; non-immune IgG (IgG): negative control for IP. The efficiency of the Co-IP can be estimated by the comparison of $\beta 1$ subunit expression in the initial total lysate (TL) and the unbound supernatant (Sup) after IP in BxPC-3 cells (Data not shown). The approximate binding efficiency was ~90 %. N=5. *, p<0.05.

Figure 4

Cell viability with co-exposure to doxorubicin and FXYD3 peptide derivatives. Cells were exposed to Dox for 48 hours and 0 (□), 1 (■) or 2 μM (●) FXYD3 peptide derivative. A. Co-exposure of BxPC-3 cells to Dox and the cysteine-mutated FXYD3-pep. B. Co-exposure of Panc-1 cells to Dox and FXYD3-pep. C. Co-exposure of MCF-7 cells to Dox and FXYD3-pep. FXYD3. D. Co-exposure of MDA-MB-468 cells to Dox and FXYD3-pep. E. Co-exposure of BxPC-3 cells to Dox and the FXYD3-pepC that retains Cys residues in the

WT FXYD3 protein. F. Co-exposure of Panc-1 cells to Dox and FXYD3-pep. Results are from 5 experiments for each FXYD3-pep concentration.

Figure 5

Na⁺/K⁺-ATPase and FXYD3 expression and distribution of FXYD3-pep. A. MCF-7 cell survival after 48 hours exposure to ouabain in concentrations indicated, with or without co-exposure to 1 μM doxorubicin (Dox). N = 5. B. FXYD3 and Na⁺/K⁺-ATPase α1 subunit expression in MCF-7 and MDA-MB-468 breast cancer cells and in BxPC-3 and Panc-1 pancreatic cancer cells. Expression is normalized to expression in human non-cancer MCF-10A cells; GAPDH was the internal loading control. N = 5; *, p<0.05. C. Electrogenic Na⁺/K⁺-ATPase pump currents (Ip) of MCF-7 cells that do- and MDA468 cells that do not overexpress Na⁺/K⁺-ATPase. The trace of membrane current shown was recorded in a MCF-7 cell before and after Na⁺-K⁺ pump activity was eliminated with exposure to K⁺-free extracellular solution. The inward shift to a near-zero holding current in K⁺-free solution identifies Ip. Currents were sampled with an electronic cursor after electrical noise caused by extracellular solution change had subsided. N = 4 for each cell line. D. Immunofluorescence showing distribution of TRITC-labeled FXYD3-pep in the BxPC-3 pancreatic cancer cells. Cells were exposed to FXYD3-pep labeled with TRITC (red) for 2 hours and fluorescence microscopy performed 24 hours after the peptide was washed off. DAPI was used to

counter-stain the nucleus (blue). One of 5 similar experiments is shown. Fluorescence is predominantly peri-nuclear. Scales are shown in the middle panel.

Figure 6

GSTπ, p53 and caspase 3/7 with suppression of FXYD3. A. Exposure of BxPC-3 cells to 1 μM FXYD3-pep for 24 hours. B. Exposure to 1 μM full length cysteine-mutated FXYD3 (FL) or FXYD3-pep (pep) for 24 hours. C indicates unexposed control. C. Summary of decrease in GSTπ with exposure to FXYD3-pep or FL in all 5 experiments shown in panels A and B. D. Transfection of siRNA with and without subsequent exposure to Dox. Cells were exposed or not exposed to FXYD3 siRNA for 48 hours as indicated and then exposed or not exposed (c) to 1 μM Dox for 48 hours. One of two similar experiments performed is shown. E. Expression of p53 in BxPC-3 and MCF-7 cells exposed (pep) or not exposed (c) to 1 μM FXYD3-pep for 24 hours and then for a further 48 hours to Dox, 0.5 μM for the BxPC-3 cells and 2.5 μM for the MCF-7 cells, with or without FXYD3-pep. N = 5, *, p<0.05. F. Caspase 3/7 activity in BxPC-3 and MCF-7 cells exposed to FXYD3-pep and Dox according to the protocol for exposures in E. N = 5, *, p<0.05.

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