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Pro-survival factor EDEM3 confers therapy resistance in prostate cancer

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Abstract: Prostate cancer is the most common cancer in men, and it is primarily driven by androgen steroid hormones. The glycosylation enzyme EDEM3 is controlled by androgen signalling and important for prostate cancer viability. EDEM3 is a mannosidase that trims mannose from mis-folded glycoproteins, tagging them for degradation through endoplasmic reticulum associated degradation. Here, we find that *EDEM3* is upregulated in prostate cancer, and this is linked to poorer disease-free survival. Depletion of EDEM3 from prostate cancer cells induces an ER stress transcriptomic signature, and EDEM3 overexpression is cyto-protective against ER stressors. EDEM3 expression also positively correlates with genes involved in the unfolded protein response in prostate cancer patients and its expression can be induced through exposure to radiation. Importantly, the overexpression of EDEM3 promotes radio-resistance in prostate cancer cells and radio-resistance can be reduced through depletion of EDEM3. Our data thus implicate increased levels of EDEM3 with a role in prostate cancer pathology and reveal a new therapeutic opportunity to sensitise prostate tumours to radiotherapy.

1. Introduction

Prostate cancer is the leading cause of cancer related deaths amongst men globally¹. To improve clinical management of the disease, there is a pressing need to understand the biological processes which underpin prostate cancer disease progression and response to chemotherapeutic agents. In its early stages, prostate cancer growth is dependent upon androgen receptor signalling, hence androgen biology has been a major focus in the field to date. We recently identified a clinically relevant, androgen regulated gene signature. In particular, glycosylation is a target for androgen control in prostate cancer². Glycosylation is the enzymatic addition of glycans to target substrates, and aberrant patterns of glycosylation have been identified in multiple human malignancies³⁻⁷. Changes to the glycome (the complete pattern of glycan modifications present on a cell or tissue) have recently been shown to be an important feature of prostate carcinogenesis, and enzymes which control these changes are exciting new therapeutic targets and biomarkers for the disease⁸⁻¹⁶.

Androgens regulate glycosylation in prostate cancer by regulating the gene expression of enzymes responsible for building and modifying glycans^{2,10}. In our previous work we identified *ER Degradation Enhancing Alpha-Mannosidase Like Protein 3 (EDEM3)* as an androgen regulated gene in prostate cancer². *EDEM3* encodes a member of the glycosyl hydrolase family of proteins, and is one of three EDEM paralogs (alongside *EDEM1* and *EDEM2*)¹⁷⁻¹⁹. The EDEM proteins are key members of the endoplasmic reticulum-associated degradation pathway (ERAD), a process responsible for degradation of mis-folded proteins as a mechanism to protect the cell from ER stress^{19,20}. This process, if activated by the accumulation of mis-folded proteins in the ER, promotes the unfolded protein response (UPR)²¹. Triggering of the UPR stimulates three key signalling pathways, mediated by the three stress sensors ATF6, PERK and IRE-1²². The UPR is an adaptive response, often observed in cancer cells, in response to ER stress through various causes, such as hypoxia, DNA damage and metabolic strain. The purpose of the UPR is to protect the cell, however when hyper-activated it can lead to cell death. Therapies such as chemotherapeutic agents and radiotherapy induce ER stress, resulting in cancer cell death and recently, proteins involved in the ER stress response have become attractive therapeutic targets to sensitise cancer patients to these types of treatment²³⁻²⁵. Although *EDEM3* has been linked to cell survival, how it does this has not been well understood. To understand the importance of *EDEM* genes in prostate cancer, we performed transcriptomic analysis of prostate cancer cell lines and patient tissue. This established *EDEM3* as the clinically significant *EDEM* paralog upregulated in prostate tumours and revealed *EDEM3* expression is associated with activity of the UPR pathway. Using *in vitro* ER stress and radiosensitivity assays, we show *EDEM3* is a pro-survival factor, that is preferentially upregulated as a protective measure in response to ER stress. Consistent with this, *EDEM3* is induced following exposure to radiation, and high levels of *EDEM3* can confer radio-resistance to cancer cells (an effect which can be reversed by targeting *EDEM3*). Our findings implicate increased levels of *EDEM3* in prostate cancer pathology and identify a new therapeutic opportunity to sensitise prostate tumours to radiotherapy.

2. Results

2.1. EDEM3 up-regulation is associated with a poor disease-free survival in prostate cancer

We first analysed The Cancer Genome Atlas (TCGA) prostate adenocarcinoma (PRAD) Firehose cohort to compare *EDEM1*, *EDEM2*, and *EDEM3* gene expression data in prostate adenocarcinoma tissue (N=497) and normal prostate tissue (N=52)²⁶. *EDEM3* expression was the only significantly upregulated *EDEM* gene in prostate tumour tissue

compared to normal tissue ($p = 0.0009$) (figure 1A). Of interest, we observed large variation in *EDEM3* expression levels in this cohort.

We next sought to validate these findings in 4 other independent patient cohorts. We carried out a meta-analysis of 366 prostate tissue samples from four different studies^{27–30} (figure 1B). Our data shows that *EDEM3* expression alone was consistently upregulated across our meta-analysis, being found, on average, in the top 2.25% of overexpressed genes in all four cohorts. *EDEM1* and *EDEM2* were found to be significantly overexpressed in only two of the four cohorts. Fold change data can be found in supplemental table 1. Our data suggests that across five independent clinical cohorts, *EDEM3* is the most highly expressed *EDEM* gene in prostate cancer and is the only *EDEM* paralog that is consistently upregulated in prostate cancer patient tissue.

To investigate the clinical implications of *EDEM* gene upregulation, we looked at *EDEM1/2/3* mRNA expression in relation to disease free survival in 471 prostate cancer patients in the TGCA PRAD cohort²⁶ (figure 1C-E). Survival analysis showed that high levels of *EDEM3* expression were significantly associated with a poorer disease-free survival rate ($p = <0.0001$). Importantly, we found no significant association between *EDEM3* mRNA expression and other clinical parameters which may affect survival (supplemental figure 1). In contrast, neither *EDEM1* nor *EDEM2* gene expression correlated with disease free survival in prostate cancer patients. These data show that *EDEM3* is the most clinically relevant *EDEM* paralog in prostate cancer.

We have previously shown *EDEM3* gene expression levels to be reduced in men post-androgen deprivation therapy. We next tested which *EDEM* genes are androgen responsive. We treated LNCaP (prostate adenocarcinoma cells) and RWPE-1 cells (normal prostatic epithelial cells) with 10 nM synthetic androgens (R1881) for 24 hours and assessed gene expression of *EDEM1*, *EDEM2* and *EDEM3* (figure 1F and figure 1G). *EDEM3* mRNA levels significantly increased following stimulation with synthetic androgens, compared with steroid depleted controls (LNCaP $p = 0.03$; RWPE-1 $p = 0.05$). In contrast, there was no consistent expression change for either *EDEM1* or *EDEM2* in response to androgens. The regulation of *EDEM3* by androgens was further confirmed by depleting LNCaP cells of the AR (figure 1H), which significantly decreased the levels of *EDEM3* ($p = 0.02$), whilst no statistically significant differences were detected for *EDEM1* or *EDEM2*.

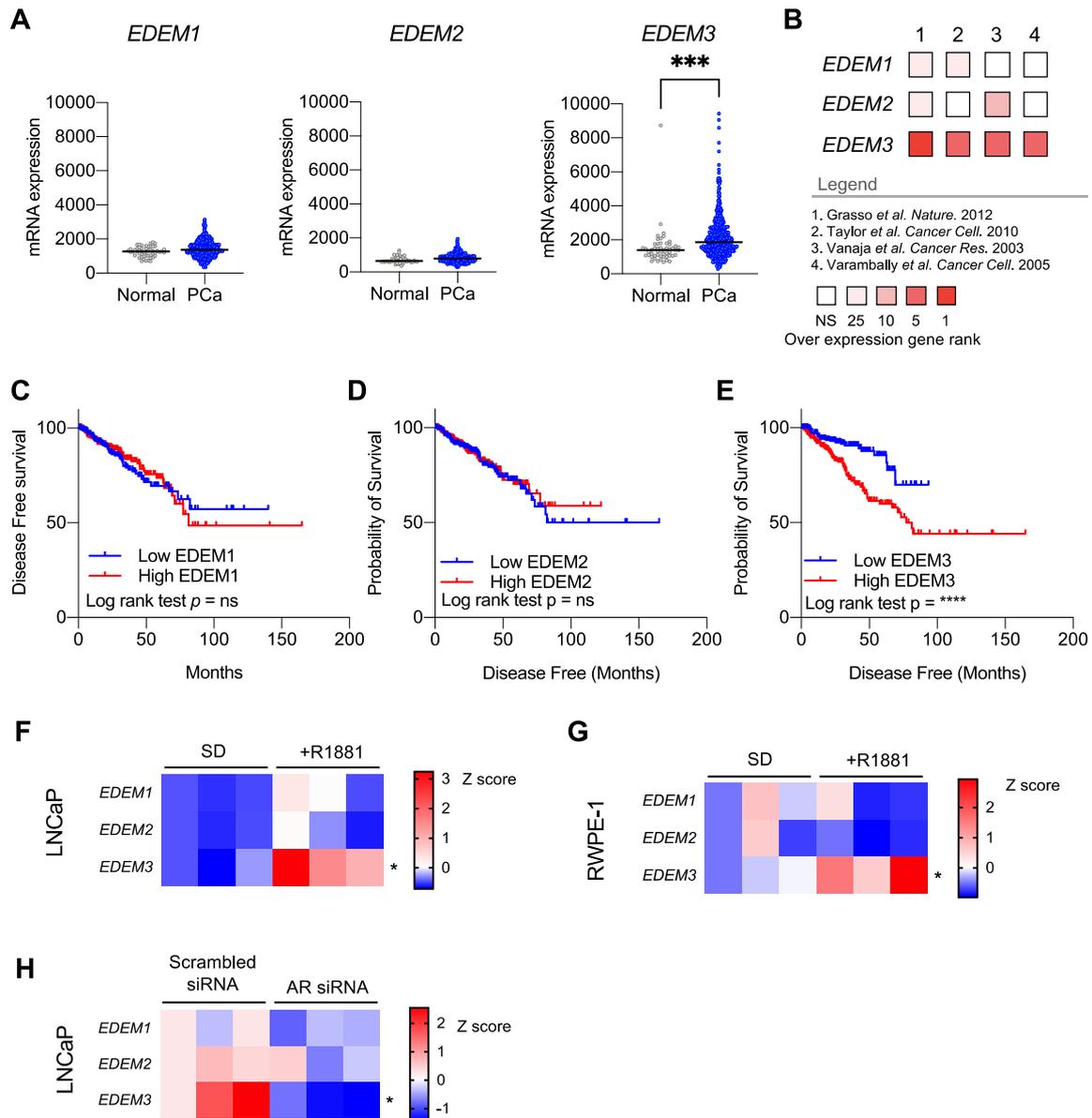


Figure 1. EDEM3 up-regulation is associated with a reduced disease-free survival in prostate cancer (PCa). (A) Analysis of *EDEM* gene expression in The Cancer Genome Atlas (TCGA) prostate adenocarcinoma cohort (N=497) compared with normal prostate samples (52). (B) Meta-analysis of *EDEM1/2/3* gene expression in prostate cancer. Four independent prostate cancer gene expression data sets (N=366) were analysed for *EDEM1/2/3* gene expression in prostate cancer patients compared with patients with normal. Data shown is of overexpression gene rank in each dataset. Legend includes details of the independent cohorts. NS = not significant, 25 = top 25%, 10 = top 10%, 5 = top 5% and 1 = top 1%. Data accessed using OncoPrint²⁷⁻³⁰. (C-E) Kaplan-Meier plot showing disease free survival for prostate cancer patients stratified based on low (bottom 50%) or high (top 50%) *EDEM3* expression. Analysis includes 471 prostate cancer patients from TCGA PRAD cohort, accessed via CBioPortal. P value was calculated by log rank test. (F-G) Heatmaps showing mRNA levels of *EDEM1/2/3* in both LNCaP and RWPE-1 cultured in either steroid depleted (SD) conditions or stimulated with 10 nM R1881 for 24 hours. N=3 and data are presented as z-scores. (H) Heatmap showing mRNA levels of *EDEM1/2/3* in LNCaP cells following androgen receptor siRNA knockdown, compared with scrambled siRNA control. N=3 and data are shown as z-scores. P values were calculated using a two tailed unpaired t-test. *P < 0.05, **P < 0.01 and ***P < 0.001.

2.2. Depletion of *EDEM3* from prostate cancer cells increases expression of ER stress and apoptosis associated genes

Each of the above data indicate that *EDEM3* is the most clinically important *EDEM* paralog in prostate cancer. As such, we focussed on further understanding the role of *EDEM3* in prostate cancer biology. To interrogate the functional effect of *EDEM3* in prostate cancer, we next generated stable prostate cancer cell lines with knockdown of *EDEM3*. We achieved approximately 70-75% *EDEM3* gene knockdown in both LNCaP and CWR22Rv1 cells and confirmed loss of *EDEM3* at the protein level (figure 2A-B).

We previously found that loss of *EDEM3* in prostate cancer cells results in a significant decrease in cellular viability ². To understand why loss of *EDEM3* in prostate cancer cells reduces cell survival, we performed RNA-sequencing of our CWR22Rv1 shRNA stable cell line. Differential gene expression analysis indicated that loss of *EDEM3* resulted in significant downregulation of 279 genes and significant upregulation of 391 genes based on an adjusted *p*-value of <0.05 (figure 2C, supplemental table 3). Gene ontology (GO) analysis of differentially expressed genes revealed gene expression changes in processes linked to stress responses (supplemental table 4). These included significant changes in ER, UPR and apoptosis associated processes, including ER protein targeting (FDR <0.0001), and ER-nucleus signalling (FDR = <0.0001). Of interest, both the 'unfolded protein response' (FDR = 0.019), and the 'PERK-mediated unfolded protein response' (FDR = 0.002) were identified as altered processes following loss of *EDEM3* in prostate cancer cells. Our data also shows changes in cell death associated process, such as 'regulation of cell death' (FDR = 0.001) and 'regulation of programmed cell death' (FDR = 0.002). Figure 2D shows a heatmap of gene expression changes which are associated with ER stress and apoptosis-associated GO terms. These differentially expressed genes include *XBP1* (adjusted *p* = 0.01) ³¹ and *ATF4* (adjusted *p* = <0.0001) ³² *CHAC1* (adjusted *p* = 0.02) ³³ and *BBC3* (adjusted *p* = 0.03) ³⁴. We next tested whether *EDEM3* depletion in a second prostate cancer cell line would promote similar gene expression changes in UPR and apoptosis associated genes. Using qPCR, we show that downregulation of *EDEM3* in LNCaP cells increases the expression of genes with roles in ER stress/UPR (*EIF2AK3*, *XBP1*, *DDIT3*, *CREB3L2* and *HERPUD1*) and apoptosis (*CDKN2A*, *ATF3*, *DDIT4* and *BBC3*) (figure 2E). This is consistent with our previous study where loss of *EDEM3* reduced prostate cancer cell viability ². To confirm that loss of *EDEM3* results in an increase in ER stress in prostate cancer cells, we measured expression of GRP78, a common marker of ER stress (figure 2F). Western blot analysis of GRP78 confirmed that upon loss of *EDEM3*, LNCaP cells have higher levels of ER stress. These findings suggest that depletion of *EDEM3* leads to an upregulation of ER stress and pro-apoptotic pathways.

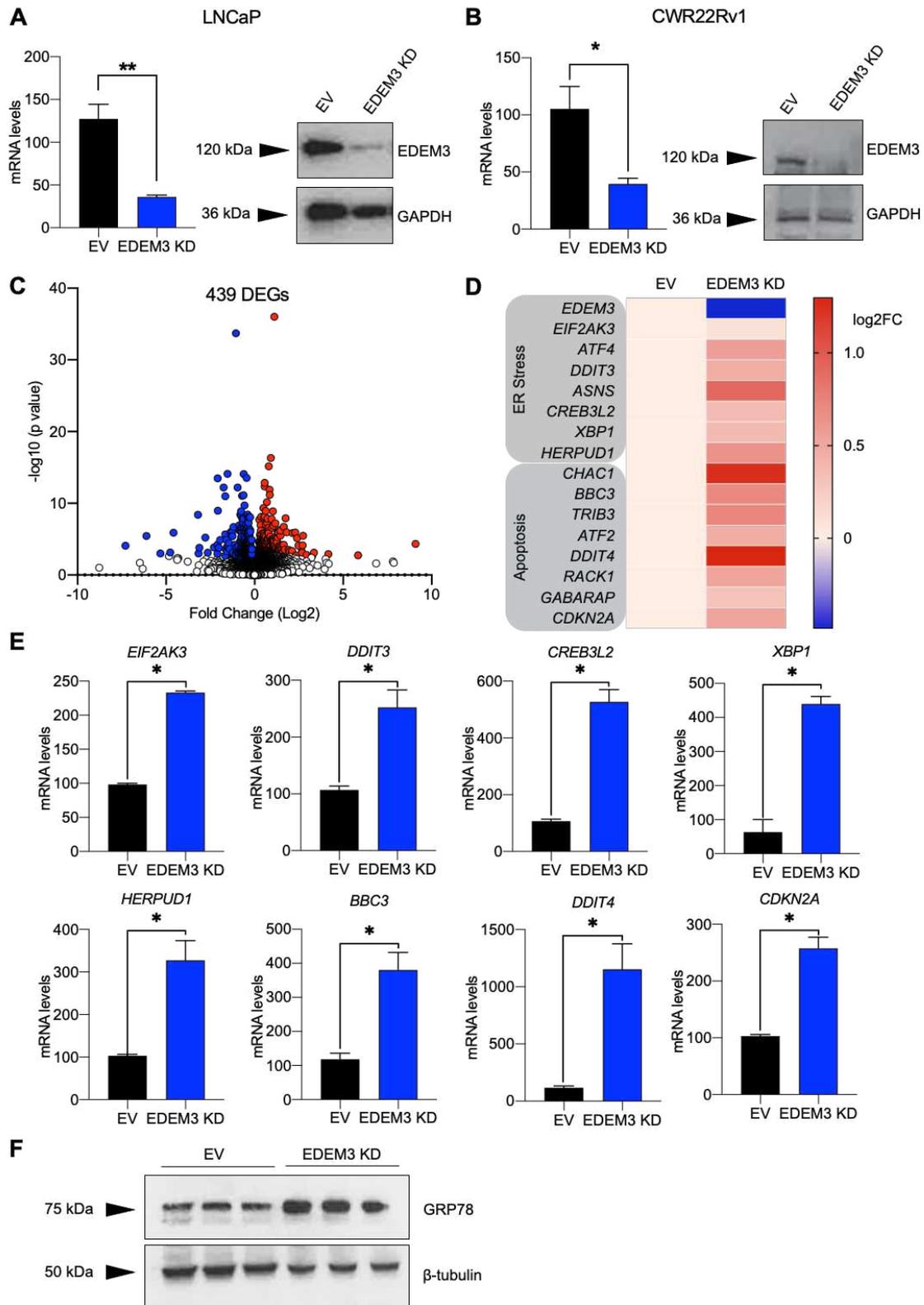


Figure 2. Depletion of EDEM3 from prostate cancer cells increases expression of ER stress and apoptosis associated genes. (A-B) mRNA and protein levels of EDEM3 in LNCaP and CWR22Rv1 cells following shRNA mediated *EDEM3* gene knockdown compared with empty vector control. mRNA levels shown as mean \pm s.e.m. Protein levels detected by western blotting. GAPDH was used as a loading control. (C) Volcano plot showing transcriptomic analysis of CWR22Rv1 cells following *EDEM3* gene knockdown. Significantly downregulated genes are shown in blue and significantly upregulated genes are shown in

red. Significance was determined using an adjusted p-value <0.05. (D) Heatmap showing the log fold change in gene expression levels of ER stress and apoptosis associated genes from RNA sequencing of CWR22Rv1 cells following EDEM3 knockdown. (E) mRNA expression levels of ER stress associated genes and pro-apoptotic genes following EDEM3 knockdown in LNCaP cells. N=3 and data are mean \pm s.e.m. (F) Western blot analysis of GRP78 in LNCaP cells following EDEM3 gene knockdown.

2.3. *EDEM3* is associated with ER stress in prostate cancer patients and is induced by ER stressors

As loss of EDEM3 in our cell line models resulted in an increase in a UPR gene signature, and the ER stress marker GRP78, we next investigated whether EDEM3 is associated with ER stress in prostate cancer patients. Many pathological features of solid tumours, such as hypoxia and oxidative toxicity can disrupt ER homeostasis, resulting in increased ER stress and activation of the unfolded protein response^{35,36}. We tested whether levels of *EDEM3* expression correlate with UPR stress sensors in prostate cancer patient tissue. Strikingly, spearman correlation analysis from the TCGA PRAD cohort (n=493)²⁶ showed that *EDEM3* expression positively correlates with each of the UPR stress sensors; *ERN1* (IFE-1 α), *EIF1AK3* (PERK) and *ATF6* (figures 2A-C). This strongly indicates that *EDEM3* expression is associated with the UPR in prostate cancer clinical tissue.

Having already shown that loss of EDEM3 results in an induction of ER stress, we wanted to investigate whether EDEM3 itself is responsive to ER stress. We treated LNCaP and CWR22Rv1 cells with the ER stressing agents thapsigargin and tunicamycin for 24 hours. Tunicamycin and thapsigargin are well established models of ER stress induction *in vitro* and act through two distinct pathways. Tunicamycin works by inhibiting N-glycosylation and thapsigargin by inhibiting calcium signalling. In the literature varying culture conditions have been used, including different timepoints, inhibitor concentrations and serum levels. Several studies have used the inhibitors on prostate cancer cell lines, and we have used these studies to select our culture conditions for these experiments³⁷⁻⁴². Following treatment with tunicamycin for 24 hours, we detected levels of the ER stress marker GRP78 and EDEM3 using western blotting. Our data confirms that treatment with tunicamycin induced ER stress in both LNCaP and CWR22Rv1 cells as shown by an increase in GRP78 protein levels (figure 3 D-E). In response to tunicamycin mediated ER stress, we detected increased levels of EDEM3 in both CWR22Rv1 and LNCaP cells. Similarly, we confirmed that thapsigargin induced ER stress in our model, shown through an increase in GRP78 protein levels (figure 3 F-G). We also detected an increase in EDEM3 in response to thapsigargin exposure (figure 3 F-G). Taken together, these data show that *EDEM3* is correlated with ER stress markers in prostate cancer patients, and that it is upregulated in response to ER stress in prostate cancer cells.

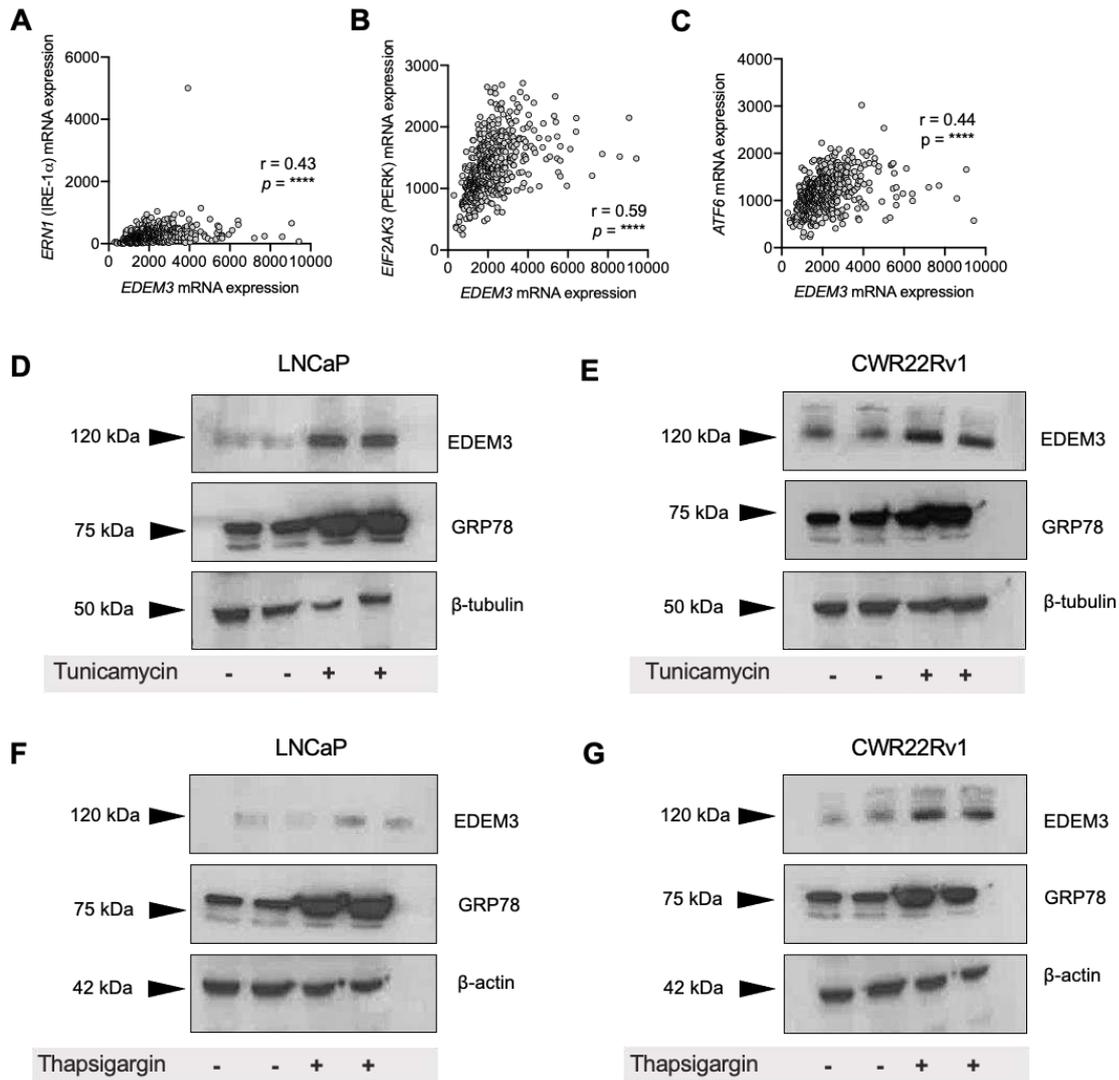


Figure 3. EDEM3 is associated with ER stress in prostate cancer patients and is ER stress responsive. (A-C) Spearman correlation of EDEM3 gene expression with the three UPR stress sensors ERN1 (IRE-1 α), EIF2AK3 (PERK) and ATF6. Gene expression values are a part of TCGA prostate adenocarcinoma (PRAD) cohort, accessed through CBioPortal. N=493²⁶. mRNA expression values for EDEM3 were correlated with ERN1, EIF2AK3 and ATF6. P values were calculated using a two-tailed spearman correlation with 95% confidence intervals. *P < 0.05, **P < 0.01 and ***P < 0.001. (D) Western blot analysis of EDEM3 and GRP78 in LNCaP cells in response to 2.5 μ g/mL tunicamycin for 24 hours. β -tubulin was used as a loading control. (E) Western blot analysis of EDEM3 and GRP78 in CWR22Rv1 cells in response to 2.5 μ g/mL tunicamycin for 24 hours. β -tubulin was used as a loading control. (F) Western blot analysis of EDEM3 and GRP78 in LNCaP cells in response to 100 nM thapsigargin for 24 hours. β -actin was used as a loading control. (G) Western blot analysis of EDEM3 and GRP78 in CWR22Rv1 cells in response to 100 nM thapsigargin for 24 hours. β -actin was used as a loading control.

2.4. EDEM3 overexpression in prostate cells protects cells from ER stressors

As EDEM3 is upregulated in prostate cancer tissue, we next created cell line models with overexpression of EDEM3. Both CWR22Rv1 and LNCaP cells were transfected with an EDEM3 expression vector or an empty vector control. In LNCaP cells we achieved an approximately four-fold overexpression and in CWR22Rv1 an approximately ten-fold

overexpression as observed at the mRNA level by qPCR. EDEM3 protein overexpression was confirmed by western blot (figure 4 A-B).

To understand the role of EDEM3 in the ER stress response in prostate cancer, we treated EDEM3 overexpressing cells with the ER stressing agents thapsigargin and tunicamycin for 24 hours. In both LNCaP and CWR22Rv1 empty vector (EV) control cells, thapsigargin significantly decreased cellular viability (LNCaP $p = 0.002$; CWR22Rv1 $p = 0.005$). This effect was however ameliorated by EDEM3 overexpression, where although there was a decrease in viability it was no longer significant (LNCaP $p = 0.16$; CWR22Rv1 $p = 0.13$) (figure 4C and 4D). In LNCaP cells treated with tunicamycin, we observed a statistically significant decrease in viability between EV control cells following treatment (figure 4E). When comparing EDEM3 overexpressing cells, tunicamycin treatment reduced cellular viability, this was not statistically significant, (figure 4E). In CWR22Rv1 cells treated with tunicamycin, although there was a significant reduction in viability in EDEM3 overexpressing cells following tunicamycin treatment, cellular viability was significantly higher with EDEM3 overexpression compared with EV control ($p = 0.04$) (figure 4F). Although we only observed a significant increase in viability in tunicamycin treated cells overexpression EDEM3 in our CWR22Rv1 cell line, EDEM3 overexpression in LNCaPs exhibited a smaller response, perhaps due to intrinsic differences in these two models.

Next, we used these models to investigate the effect of EDEM3 overexpression on ER stress and pro-apoptotic gene signatures. We used qPCR to monitor a panel of 15 genes linked to ER stress and apoptosis that were also previously identified as being differentially expressed following EDEM3 knockdown in our RNA sequencing screen. In LNCaP cells, EDEM3 overexpression resulted in a significant downregulation of several ER stress and apoptotic genes, including *EIF2AK3*, *XBP1*, *BBC3* and *RACK1* (figure 4G). EDEM3 overexpression in CWR22Rv1 cells also led to a significant decrease in ER stress and apoptosis related genes, including *EIF2AK3*, *HERPUD1*, *BBC3* and *CHAC1* (figure 4H). This suggests that overexpression of EDEM3 in prostate cancer cells can leads to the reduction of ER stress and apoptosis associated gene signatures.

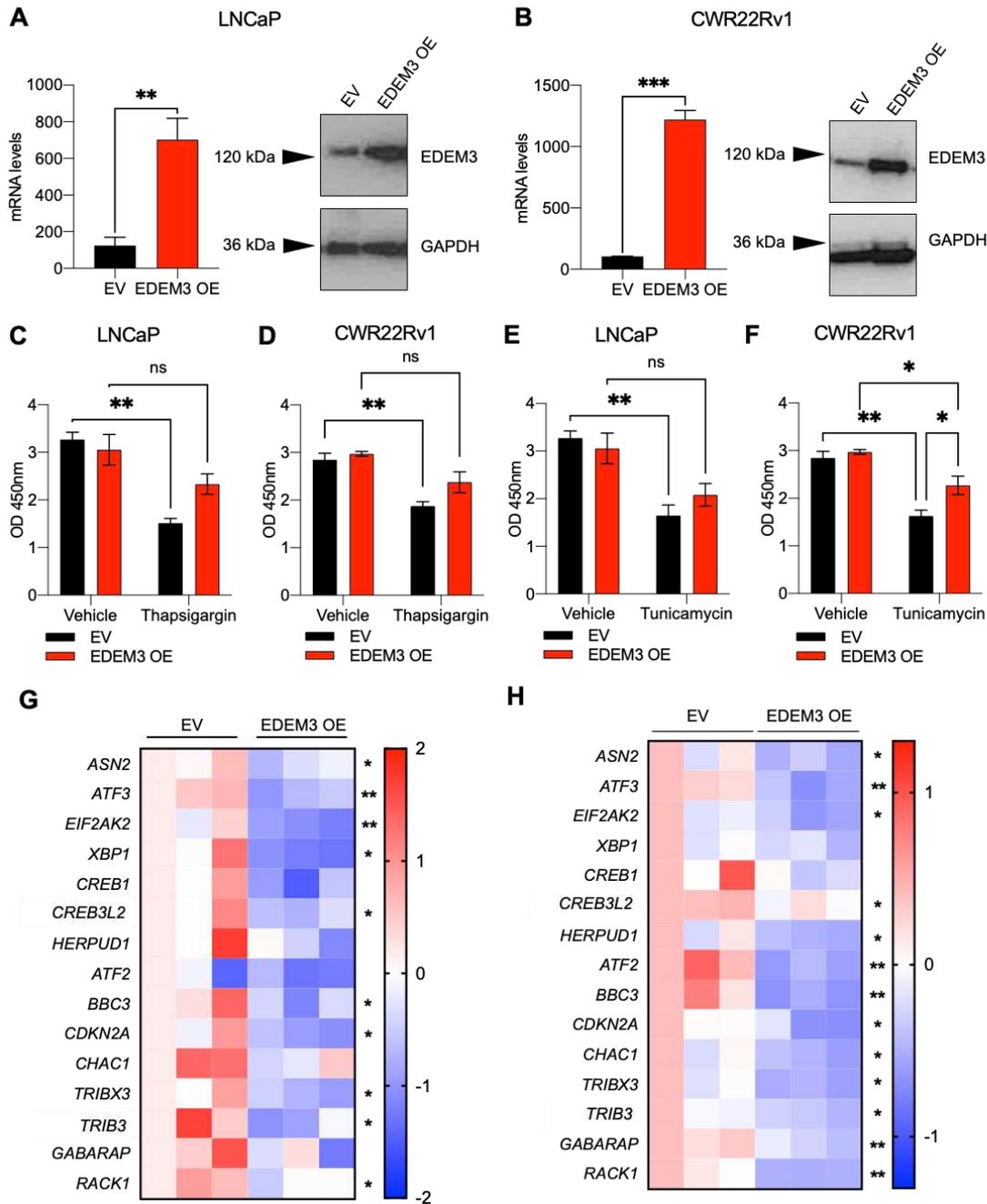


Figure 4. EDEM3 overexpression in prostate cells protects from ER stressors (A-B) mRNA and protein levels of EDEM3 in LNCaP and CWR22Rv1 cells following overexpression of EDEM3 compared with an empty vector control. Data are presented as mean \pm s.e.m. Western blot analysis shown with GAPDH used as a loading control. (C-F) Cellular viability of LNCaP and CWR22Rv1 cells, with overexpression of EDEM3, treated with either vehicle (DMSO), 2.5 μ M tunicamycin or 100 nM thapsigargin for 24 hours. Viability measured by WST-1 assay and shown as absorbance at O.D 450 nm. N=3. Data are mean \pm s.e.m. *P* values were calculated using two tailed unpaired t-tests. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. (G-H) Heatmap showing mRNA levels of *ASN2*, *ATF3*, *EIF2AK3*, *XBP1*, *CREB1*, *DDIT3*, *HERPUD1*, *ATF2*, *BBC3*, *CDKN2A*, *CHAC1*, *TRIBX3*, *DDIT4*, *GABARAP* and *RACK1* in LNCaP (G) and CWR22Rv1 (H) cells following overexpression of EDEM3 compared with empty vector control. N=3 and data are presented as z-scores. *P* values were calculated using two tailed unpaired t-tests. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

2.5. Loss of *EDEM3* sensitises prostate cancer cells to ER stressors, and its upregulation promotes radio-resistance

Having shown that in our model, *EDEM3* overexpression can protect cancer cells from ER stress inducing agents, we hypothesised that targeting *EDEM3* may sensitise cancer cells to the ER stressors tunicamycin and thapsigargin.

LNCaP and CWR22Rv1 cells with depleted *EDEM3* were treated with tunicamycin (2.5 µg/mL) and thapsigargin (100 nM) (figure 5 A-D). In both LNCaP and CWR22Rv1 cells, treatment with either thapsigargin or tunicamycin resulted in a significant decrease in cellular viability, and this proved significantly more deleterious to cells with *EDEM3* gene knockdown (LNCaP thapsigargin $p = 0.03$; LNCaP tunicamycin $p = 0.003$; CWR22Rv1 thapsigargin $p = 0.03$; CWR22Rv1 tunicamycin $p = 0.04$). These data suggest that knockdown of *EDEM3* sensitises prostate cancer cells to ER stress.

Radiotherapy is often used in the clinical management of prostate cancer. DNA damage has been shown to activate members of the UPR in glioblastoma, breast cancer and colorectal cancers^{43,44}. There is also broad evidence that overexpression of members of the UPR results in resistance to radiotherapy^{45,46}. More recently, it has become clear that targeting the UPR may present an attractive opportunity to overcome radio-resistance^{24,25}. As our data above showed that *EDEM3* expression levels are very closely associated with the UPR, we sought to test whether exposure to radiation could also induce *EDEM3* expression. To test this, we exposed LNCaP cells to radiation and analysed *EDEM3* gene and protein expression. A radiation dose of 4 Gy was selected based on previous studies with this cell line^{47,48}. At 48 hours post irradiation, we observed a 60% increase in *EDEM3* gene expression and an increase in *EDEM3* protein expression as detected by western blot (figure 5E and 5F).

Given the induction of *EDEM3* expression in irradiated cells, we sought to establish the effect of *EDEM3* overexpression on cellular viability in response to radiation exposure. We therefore performed clonogenic assays on both LNCaP and CWR22Rv1 cells overexpressing *EDEM3* following exposure to either 2 or 4 Gy radiation (figure 5G and 5H). An increase in cellular viability was observed in irradiated LNCaP and CWR22Rv1 cells with upregulated *EDEM3* compared to control cells, suggesting that high levels of *EDEM3* might confer radio-resistance to prostate cancer cells. As a proof-of-concept experiment to show that targeting the UPR via *EDEM3* sensitises cells to radiotherapy, we subjected LNCaP and CWR22Rv1 cells with two doses (2 and 4 Gy) radiation after *EDEM3* depletion (figure 5I and 5J). Here, we observed a significant decrease in cell viability in irradiated cells with loss of *EDEM3*, with both LNCaP and CWR22Rv1 cell lines exhibiting a dramatic 50% reduction in cell viability with exposure to 4 Gy radiation (LNCaP $p = 0.001$; CWR22Rv1 $p = 0.0003$).

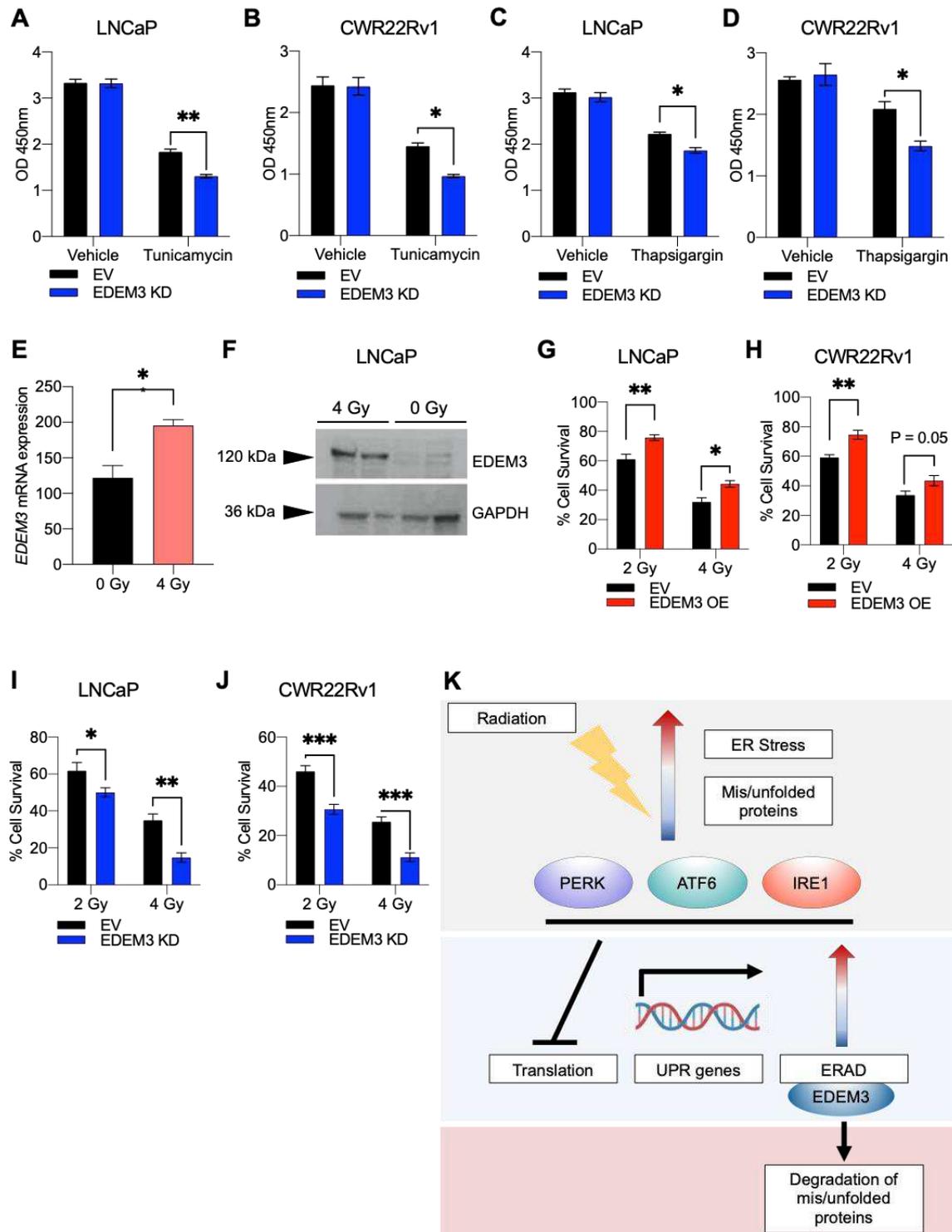


Figure 5. EDEM3 depletion in prostate cancer sensitises cells to ER stress and its upregulation promotes radio-resistance (A-D) Cellular viability of LNCaP and CWR22Rv1 cells, with knockdown of EDEM3, treated with either vehicle (DMSO), 100 nM thapsigargin or 2.5 μ g/mL tunicamycin for 24 hours. Viability measured by WST-1 assay and shown as absorbance at O.D 450 nm. N=3. Data are mean \pm s.e.m. P values were calculated using two tailed unpaired t-tests. *P < 0.05, **P < 0.01 and ***P < 0.001. (E) mRNA levels of EDEM3 in LNCaP cells 48 hours after exposure to 4 Gy radiation. Data are presented as mean \pm s.e.m. P value calculated using a two-tailed unpaired t-test. *P < 0.05. (F) Western blot analysis of EDEM3 expression in LNCaP cells 48 hours after exposure to 4 Gy radiation. GAPDH was used as a loading control. (G-H) Cell

survival determined by clonogenic assay. LNCaP and CWR22Rv1 cells transfected with either an empty vector or EDEM3 overexpression vector were irradiated with either 2 or 4 Gy and left to form colonies for 14 days. At 14 days, any colony with more than 50 cells was counted. Cell survival was calculated by comparison with relative 0 Gy controls. N=5. Data are mean \pm s.e.m. P values were calculated using a two-way ANOVA. *P < 0.05, **P < 0.01 and ***P < 0.001. (I-J) Cell survival determined by clonogenic assay. Stable EDEM3 knockdown and control LNCaP and CWR22Rv1 cells were irradiated with either 2 or 4 Gy and left to form colonies for 14 days. At 14 days, any colony with more than 50 cells was counted. Cell survival was calculated by comparison with relative 0 Gy controls. N=5. Data are mean \pm s.e.m. P values were calculated using a two-way ANOVA. *P < 0.05, **P < 0.01 and ***P < 0.001. (K) Model of the role of EDEM3 in radio-resistance. Exposure to radiation induces ER stress and an accumulation of misfolded and unfolded proteins in cells. In response, the UPR is activated by three critical sensing proteins, PERK, ATF6 and IRE1. Activation of the UPR results in the inhibition of translation, transcription of UPR associated genes, and stimulation of ERAD. EDEM3, an important ERAD associated enzyme, begins to trim mannose from misfolded glycoproteins to signal for degradation, aiding cell survival, and protecting cells from radiation.

3. Discussion

EDEM3 is an EDEM protein paralog which plays a role in mannose trimming to promote the degradation of misfolded glycoproteins. Very few studies have been conducted examining the role of EDEM3 in human disease, and we were the first to implicate EDEM3 in human malignancies². Here, using *in vitro* functional assays and transcriptomic analysis, we identify EDEM3 as an important ERAD and UPR associated gene, responsible for ensuring protection from ER stress in prostate cancer cells. Our data suggests that EDEM3 acts as a pro-survival factor in prostate cancer, and that upregulation of EDEM3 may protect cells from ER stress and have implications for radio-resistance in prostate cancer.

The data presented in this study suggest that *EDEM3* is the EDEM gene, which is preferentially upregulated in prostate cancer, showing consistent upregulation across 863 prostate cancer patients. Although it is consistently upregulated, we did observe large variation in its mRNA expression in patients, perhaps linked to the well documented heterogeneity of prostate tumours⁴⁹. *EDEM1* and *EDEM2* were slightly increased in prostate cancer patients in two out of five cohorts, suggesting that they may play some role in ERAD in prostate cancer, however their expression was in no way associated with disease free progression. High *EDEM3* expression however was associated with a poor disease-free progression in patients. This is in line with our previous work suggesting that EDEM3 promotes cancer cell viability.

Transcriptomic analysis of EDEM3 knockdown cells, presented here, has corroborated a role for EDEM3 as a pro-survival factor, showing that following EDEM3 knockdown, expression levels of pro-apoptotic and UPR genes increase. Whilst we see an increase in transcription of pro-apoptotic genes, we did not observe a decrease in viability in cells not exposed to a stressing agent. As much of the literature around EDEM3 focuses on its role as an ERAD associated enzyme, cells may need to be in a stressed condition before a loss of EDEM3 translates to a decrease in viability. In 2006, Hirao *et al.* established a role for EDEM3 in the quality control process, responsible for recognising and correctly disposing of misfolded glycoproteins, with a specific α 1,2-mannosidase activity, distinct from the mannose trimming

capabilities exhibited by other EDEM paralogs²⁰. Protein mis-folding and ER stress are byproducts of stress inducing conditions in tumours such as hypoxia, nutrient deficits, high metabolic demand and oxidative stress^{50–52}. The UPR and ERAD are important pro-survival pathways which tumours exploit to balance ER homeostasis, ensuring their continued survival and growth⁵³. Although EDEM3 had previously been identified as a vital ERAD associated protein, its role in the UPR and ERAD in cancer had yet to be explored. Our RNA-sequencing analysis supports the idea that EDEM3 is an important member of the ERAD family, identifying ER stress and the UPR as factors affecting cell survival in EDEM3 knockdown cells. Importantly, we have shown EDEM3 to be associated with ER stress using multiple stressors (tunicamycin, thapsigargin and irradiation), suggesting that independent of the method of ER stress induction, EDEM3 is linked to ERAD.

Targeting proteins involved in the UPR and ERAD have previously been shown to sensitise cells to ER stressors^{54–56}. As we have shown EDEM3 to be a cyto-protective against ER stressors, we suggest that targeting of EDEM3, shown here through gene knockdown, may be an effective strategy to sensitise cancer cells to ER stress inducing agents. Taking this further, many are now looking at the UPR and ERAD components as key mediators of resistance to common therapies such as radiotherapy and chemotherapy^{53,57,58}. Our *in vitro* data suggests that, in these models, high levels of EDEM3, as well as promoting cell survival, may promote resistance to radiation therapy. Although factors which modulate radiosensitivity have been identified, such as DNA damage and reactive oxygen species, the mechanisms of acquired radio-resistance in prostate cancer are still poorly understood^{59,60}. Further studies, including *in vivo* analyses may help to decipher specific mechanisms of radio-resistance. Future work may investigate a clinical role for EDEM3 as a therapeutic target in combination with radiotherapy for prostate cancer. Here, we present proof of concept data to show for the first time that targeting EDEM3 may provide an opportunity to reduce levels of radio-resistance, and that the role of EDEM3 as a therapeutic target for prostate cancer warrants further investigation

4. Materials and Methods

4.1. Cell Culture and generation of stable cell lines

Cell culture and the cell lines used were as described previously². LNCaP and CWR22Rv1 cell lines were maintained in RPMI-1640 medium, supplemented with 10% foetal bovine serum and 1% penicillin streptomycin. RWPE-1 cells were maintained in keratinocyte serum free media (Gibco, 17005042) supplemented with 1% penicillin streptomycin, 0.05 mg/mL bovine pituitary extract and 5 ng/mL epidermal growth factor.

For the stable knockdown of EDEM3, control and EDEM3 shRNA lentiviral particles were purchased from Santa Cruz (sc108080 and sc-78683-V). Transductions were carried out according to the manufacturer's protocol and a multiplicity of infection (MOI) of 5 was used for both shControl and shEDEM3. Successfully transduced cells were selected using 5 µg/mL puromycin.

To generate cell lines with stable overexpression of EDEM3, an EDEM3 cDNA ORF clone in a pcDNA3.1-C-(k)DYK expression vector was purchased from Genscript (clone number: OHu04410), and transfected into 0.2×10^6 LNCaP or CWR22Rv1 cells in a 6 well dish using lipofectamine 3000 according to the manufacturer's protocol. A pcDNA3.1-C-(k)DYK empty vector was used as the empty vector control. Successfully transfected cells were selected for using 5 µg/mL puromycin.

4.2. Cell treatments

Androgen stimulation experiments were carried out as described previously⁶¹. For experiments in which LNCaP and RWPE-1 cells were stimulated with androgens, cells were cultured in steroid deplete media (media supplemented with dextran charcoal stripped foetal bovine serum) for 72 hours, at which point 10nM synthetic androgen analogue (R18811) (Perkin–Elmer, NLP005005MG) was added for 24 hours. The RNA samples used for this study have been previously validated and published⁶¹.

Tunicamycin was purchased from Sigma (T7765), resuspended in DMSO and used at a concentration of 2.5 µg/mL for all experiments. Thapsigargin was purchased from Sigma (T9033) resuspended in DMSO and used at a concentration of 100nM for all experiments. Cells were stimulated with ER stressors for 24 hours before conducting cellular viability assays. Control cells were treated with DMSO as a vehicle control.

4.3. RNA sequencing

CWR22Rv1 cells with stable *EDEM3* knockdown and mock depleted cells treated with control shRNA lentivirus were used for RNA sequencing. RNA was extracted using the Qiagen RNAeasy kit (74104) according to manufacturer's protocol. The RNA sequencing was performed at Newcastle University Genomics Core Facility using TruSeq Stranded mRNA Sequencing NextSeq High-Output to obtain 2×75 bp reads. Quality control of reads was performed using FastQC. Reads were mapped to the hg38 transcriptome using Salmon. Differential gene expression analysis was performed using DESeq2. Data was further analysed using the gene ontology resource (<http://geneontology.org>)^{62,63}.

4.4. Quantitative PCR (qPCR)

Cells were harvested and RNA extracted using Tri-reagent (Invitrogen, 15596-026) according to the manufacturers protocol. CDNA synthesis was performed on 500 ng of RNA using the Superscript VILO cDNA synthesis kit (Invitrogen, 11754-050). Quantitative PCR (qPCR) was performed in triplicate using SYBR® Green PCR Master Mix (Invitrogen, 4309155) and the QuantStudio 7 Flex Real-Time PCR System (Life Technologies). Gene expression values were normalised to the average of three housekeeping genes: GAPDH, β -tubulin and actin. Primer sequences available in supplemental table 1.

4.5. Clonogenic Assays

Cells were irradiated at room temperature in a T75 flask at a dose of either 2 or 4 Gy per minute. Cells were then trypsinised and detached from the flask and then plated at an appropriate density in 100 mm dishes and maintained for 14 days until colonies of more than 50 cells/colony had formed. Cells were then fixed at room temperature in 10% formalin for 15 minutes and stained with 0.5% crystal violet for 10 minutes at room temperature. The number of colonies containing >50 cells were then counted as representative of cell survival. Cell survival was calculated as a percentage compared with non-irradiated controls.

4.6. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). Data are presented as the mean of three independent samples \pm standard error of the mean (SEM). Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Author Contributions: Conceptualization, E.S. and J.M.; methodology, E.S and J.M.; formal analysis, E.S. and K.C.; investigation, E.S., R.G., K.C. and A.D.; data curation, E.S.; writing—original draft preparation, E.S.; writing—review and editing, J.M. and D.J.E.; visualization, E.S.; supervision, E.S., J.M. and D.J.E.; project administration, J.M.; funding acquisition, J.M. and E.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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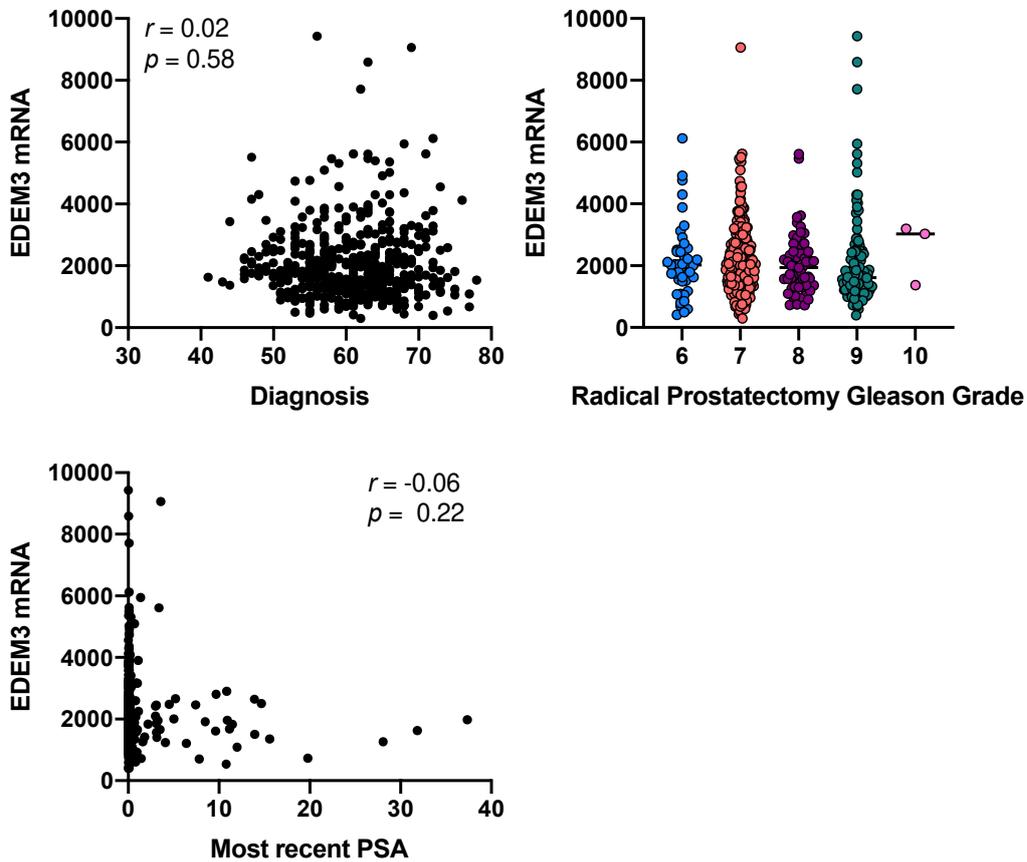
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Supplementary figures



Supplementary figure 1. The relationship between EDEM3 mRNA expression and clinical parameters in 472 patients in the TCGA PRAD cohort. EDEM3 mRNA expression does not correlate with either Age at Diagnosis or the most recent PSA score (pearson's correlation). There is no statistically significant difference in EDEM3 mRNA expression in patients with a different Gleason grades.

Supplemental information

Supplemental table 1

Antibodies details		
EDEM3	Sigma	E8906
GAPDH	Abgent	AP7873b
anti-mouse secondary	Jackson labs	715-036-150
Vectors		
shRNA control vector (LV particles)		
shEDEM3 vector (LV particles)		
Control vector	Genscript	pcDNA3.1-C-(k)DYK
EDEM3 OE vector	Genscript	OHu04410
Primers		
	Forward	Reverse
GAPDH	AAC AGC GAC ACC CAT CCT C	CAT ACC AGG AAA TGA GCT TGA CAA
ACTIN	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC
EDEM3	CAGAAAAGGCACGCAACAT	TGGAACAGAGGGGCAGTATC
ASN2	TGGCTGCTAGAAAGGTGGC	AGCCTGAATGCCTTCCTCAG
ATF3	GATTGGCTTAACCTCGGCAATAC	GACAGCCAAATGATCCTCGTTG
EIF2AK3	CTGTCACTCAGGTGGCAGC	CTCCAAGAAGGCAAGGACG
XBP1	GCGGAAGCCAAGGGGAATG	TGCACGTAGTCTGAGTGCTG
CREB3L2	TCCTGCGCTTAGCATCCTC	GTTCTGAGAAGTGCGTGTGG
DDIT3	CCTCCTGGAAATGAAGAGGAAG	GTGACCTCTGCTGGTTCTGG
HERPUD1	TGAATGCACAAGGCATCACG	ACGTCAGGAGGAGGACCATC
ATF2	GATTGGCTTAACCTCGGCAATAC	GACAGCCAAATGATCCTCGTTG
BBC3	TGCCAGATTTGTGGTCCTCAG	TACTGTGCGTTGAGGTCGTC
CDKN2A	GGGTAGAGGAGGTGCGGG	CCCATCATCATGACCTGGATCG
CHAC1	CTTGAAGATCATGAGGGCTGC	TGGTATCGTAGCCACCAAGC
TRIB3	GGTCTGTTTTGCATGCGAGC	ACTCCAACCGCTTCTTCCTG
DDIT4	GGTTTGACCGCTCCACGAG	GGTAAGCCGTGTCTTCCTCC
GABARAP	ATCCGTTTCGAGAAGCGCC	AGCCTTTTCTACTATCACCGGC
RACK1	AATACCCTGGGTGTGTGCAA	GCCAGGTTCCATACCTTGACC

Supplemental table 2

Oncomine - fold change PCa vs normal												
Gene	Grasso <i>et al.</i>			Taylor <i>et al.</i>			Varambally <i>et al.</i>			Vanaja <i>et al.</i>		
	Fold change	Gene rank	P value	Fold change	Gene rank	P value	Fold change	OE gene rank	P value	Fold change	OE gene rank	P value
EDEM1	1.158	top 21%	1.50E-02	1.09	top 14%	7.00E-03	-1.1	top 75%	NS	1.1	top 36%	9.00E-01
EDEM2	1.14	top 23%	2.40E-02	1.02	top 48%	NS	1.15	top 27%	NS	1.2	top 10%	4.59E-06
EDEM3	1.6	top 1%	2.18E-10	1.5	top 2%	6.77E-07	1.6	top 4%	3.00E-03	1.7	top 2%	4.59E-06

Supplementary Files

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