# ***FRMD6* has tumor suppressor functions in prostate cancer**

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**Keywords:** FRMD6, prostate cancer, biomarker, CRISPR/Cas9, tumor suppressor gene

# **Abstract**

Available tools for prostate cancer (PC) prognosis are suboptimal but may be improved by better knowledge about genes driving tumor aggressiveness. Here, we identified *FRMD6* (FERM domain-containing protein 6) as an aberrantly hypermethylated and significantly downregulated gene in PC. Low FRMD6 expression was associated with post-operative biochemical recurrence in two large PC patient cohorts. In overexpression and CRISPR/Cas9 knockout experiments in PC cell lines, FRMD6inhibited viability, proliferation, cell cycle progression, colony formation, 3D spheroid growth, and tumor xenograft growth in mice. Transcriptomic, proteomic, and phospho-proteomic profiling revealed enrichment of Hippo/YAP and c-MYC signaling upon *FRMD6* knockout. Connectivity Map analysis and drug repurposing experiments identified pyroxamide as a new potential therapy for *FRMD6* deficient PC cells. Finally, we established orthotropic *Frmd6* and *Pten,* or *Pten* only (control) knockout in the ROSA26 mouse prostate. After 12 weeks, *Frmd6/Pten* double-knockouts presented high-grade prostatic intraepithelial neoplasia (HG-PIN) and hyperproliferation, while *Pten* single-knockouts developed only regular PIN lesions and displayed lower proliferation. In conclusion, *FRMD6* was identified as a novel tumor suppressor gene and prognostic biomarker candidate in PC.

# **Introduction**

Prostate cancer (PC) is the most commonly diagnosed non-cutaneous malignancy and the second leading cause of cancer-related death amongst men in the Western world [[1](#_ENREF_1)]. While many PCs remain indolent throughout the patient’s lifetime, others progress to highly aggressive disease, causing significant morbidity and mortality. Routine prognostic tools cannot clearly distinguish aggressive from indolent PC, resulting in overtreatment of many clinically insignificant tumors and suboptimal treatment of aggressive tumors [[2](#_ENREF_2)].

In the present study, we aimed to identify novel molecular drivers of PC aggressiveness. By RNA-sequencing (RNA-Seq) of non-malignant (NM) *vs.* PC tissue samples as well as less *vs.* more aggressive isogenic PC cell lines, we identified *FRMD6* (FERM domain-containing protein 6) as a novel tumor suppressor gene (TSG) candidate in PC. FRMD6 belongs to the Ezrin/Radixin/Moesin (ERM) protein family and has been proposed as the human orthologue of Drosophila Expanded [[3](#_ENREF_3)]. FRMD6 can bind to actin filaments [[4](#_ENREF_4)] and nectins [[5](#_ENREF_5)], thus regulating actomyosin contractility in the cytoskeleton and epithelial cell-cell junction complexes to maintain epithelial structure [[6](#_ENREF_6)]. FRMD6 has previously been identified as an upstream regulator of the Hippo signaling cascade regulating mammalian cell growth and differentiation, including cell contact inhibition, apoptosis, proliferation, and tissue regeneration [[7](#_ENREF_7)]. Furthermore, deregulation of Hippo pathway components has been reported in various human cancers, including PC [[8-10](#_ENREF_8)].

In this study, we investigated the expression pattern, biomarker potential and biological role of *FRMD6* in PC development and progression. By analyzing transcription profiling data from >800 PC patients, we found significant downregulation of FRMD6 in PC *vs.* NM prostate tissue specimens and in metastatic *vs.* localized PC. Low FRMD6 expression was associated with aberrant promoter hypermethylation in PC tumors and with higher post-operative biochemical recurrence risk. Furthermore, functional studies in 2D, 3D, and xenografts showed that *FRMD6* knockout increased PC cell viability and proliferation, and stimulated colony formation and cell cycle progression. By integrative analysis of the transcriptome, proteome, and phospho-proteome of PC cells, we identified Hippo, mTOR and c-MYC signaling as mediators of FRMD6 and PC aggressiveness. For *in vivo* validation, we established a murine orthotopic *Frmd6/Pten* double-knockout model, where prostatic cells with combined *Frmd6/Pten* knockout displayed higher cell proliferation than *Pten* single-knockout controls. In summary, our results establish *FRMD6* as a novel tumor suppressor gene and prognostic biomarker candidate in PC.

**Results**

## FRMD6 is downregulated in PC

To identify novel genes involved in PC development and progression, we analyzed total RNA-Seq data from 18 NM and 55 PC tissue specimens (Set#1; Supplementary Table S1), one normal primary prostate epithelial cell line (PrEC) and two pairs of less/more aggressive isogenic PC cell lines (PC3/PC-3M and DU145/DU145-MN1; Supplementary Table S2). For candidate selection, we specifically searched for transcripts that were differentially expressed in NM *vs.* PC tissue specimens as well as in less *vs.* more aggressive PC cell lines. We found that FRMD6was significantly downregulated in PC *vs.* NM tissue specimens (Set#1, FDR=9.64E-08, Fig. 1A) and in the more aggressive PC-3M and DU145-MN1 cell lines *vs.* their isogenic counterparts PC3 and DU145 (Fig. 1B). These results were confirmed by RT-qPCR analysis of FRMD6expression in 28 independent patient samples (19 PC and 9 NM tissue specimens) (Supplementary Fig. S1).

For external validation, we used FRMD6 transcriptional expression data from four public PC patient sets [[11-14](#_ENREF_11)]. FRMD6 was significantly downregulated in PC *vs.* NM prostate tissue samples in The Cancer Genome Atlas (TCGA/PRAD) cohort [[11](#_ENREF_11), [15](#_ENREF_15), [16](#_ENREF_16)] (479 PC vs. 29 NM; p<0.0001; Fig. 1C), in the Taylor cohort [[12](#_ENREF_12)] (150 PC vs. 29 NM; p<0.0001; Fig. 1D), and in the Grasso cohort [[13](#_ENREF_13)] (57 PC vs. 22 NM; p<0.001; Fig. 1E). FRMD6 was further downregulated in advanced mCRPC samples (n=29) analyzed in the Grasso cohort (p≤0.0001; Fig. 1E). Moreover, in the Long cohort [[14](#_ENREF_14)], FRMD6 expression was significantly lower in PC patients with *vs.* without BCR (p=0.0027; Fig. 1F). These results indicate that PC development and progression are associated with downregulation of FRMD6and suggest a possible tumor suppressor role for *FRMD6* in PC.

## FRMD6 downregulation in PC is associated with aberrant promoter hypermethylation

To explore the mechanism(s) behind FRMD6 downregulation in PC, we investigated whether the *FRMD6* gene is subject to mutations or copy number alterations (CNAs) in PC tumors. Based on whole exome/genome sequencing and SNP/comparative genomic hybridization array data from 13 PC studies in cBioPortal ([www.cbioportal.org](http://www.cbioportal.org)), genomic alterations affecting the *FRMD6* gene were detected in only 18 out of 3441 (0.5%) primary PC samples. In contrast, DNA methylation array data available in house (33 NM and 43 PC; Set#2+3 [[15-17](#_ENREF_15)]) and from TCGA (29 NM and 497 PC samples [[18](#_ENREF_18), [19](#_ENREF_19)]) showed very common and highly PC-specific promoter hypermethylation of *FRMD6* in both patient sets with AUCs ≥0.88 in ROC curve analyses (Fig. 1G-J; Supplementary Fig. S2). There was a significant inverse correlation between expression and *FRMD6* promoter methylation levels in both patient sets (Set#3: Rho=-0.814, p<0.0001. TCGA: Rho=-0.613, p<0.0001, Fig. 1K, L), consistent with epigenetic silencing. These results indicate that *FRMD6* is a common target for aberrant promoter hypermethylation in PC, whereas somatic mutations and CNAs are rare.

## Prognostic potential of FRMD6

To investigate the prognostic potential of FRMD6 transcriptional expression, we performed Kaplan-Meier analyses of post-operative BCR in two large PC patient cohorts with sufficient clinical follow-up (Taylor [[12](#_ENREF_12), [20](#_ENREF_20)] and Long [[14](#_ENREF_14)] cohorts; Supplementary Table S3). Patients with high FRMD6 expression had significantly reduced risk of BCR in the Taylor cohort (p=0.036; Fig. 1M), while this was borderline significant in the Long cohort (p=0.056; Fig. 1N). Similarly, in univariate Cox regression analysis, high FRMD6expression was associated with significantly better BCR-free survival in both cohorts (Taylor: HR (95% CI) = 0.33 (0.14-0.78), p=0.012; Long: HR (95% CI) = 0.64 (0.43-0.96), p=0.027; Table 1). After adjustment for clinicopathological parameters in multivariate analysis, FRMD6 expression remained a significant independent predictor of BCR-free survival in both cohorts (Taylor: HR (95% CI) = 0.38 (0.16–0.88), p*=*0.024; Long: HR (95% CI) = 0.51 (0.32–0.82), p*=*0.005; Table 1). Adding FRMD6to a multivariate model of clinicopathological factors improved predictive accuracy (C-index) from 0.788 to 0.798 and from 0.706 to 0.747 in the Taylor and Long cohort, respectively. These results indicate that reduced FRMD6 expression is an adverse predictor of PC recurrence, independent of clinicopathological parameters.

## FRMD6 inhibits PC cell viability, proliferation, cell cycle progression and anchorage-independent growth *in vitro*

To explore the biological function of *FRMD6* in PC, we transfected PC-3M and DU145-MN1 cells (both have low endogenous FRMD6 expression levels; Fig. 1B) with a plasmid encoding the most predominant isoform of FRMD6. Overexpression of FRMD6 was verified by RT-qPCR and Western blotting, and lasted for at least 96h (Supplementary Fig. S3A-D). Ectopic expression of FRMD6 significantly attenuated PC-3M and DU145-MN1 cell viability and proliferation at 72h compared to mock transfected controls (p<0.05; Fig. 2A-C).

Next, we used CRISPR/Cas9 to establish PC3 and DU145 cell lines with genomic knockout (KO) of *FRMD6* (PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6) and scrambled controls (PC3\_SCR and DU145\_SCR). We chose PC3 and DU145 due to their high endogenous FRMD6 expression level (Fig. 1B). The knockout was validated by TIDE analysis, RT-qPCR and Western blotting (Supplementary Fig. S4A-C). Both cell viability and proliferation were significantly increased for both PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 as compared to scrambled controls (p<0.05; Fig. 2D-F). Transient FRMD6 rescue (validated by RT-qPCR; Supplementary Fig. S5A, B) attenuated the increased cell viability in both PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 to parental levels at 72h (p<0.005; Fig. 2G). Cell cycle analysis revealed a significantly higher proportion of PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 cells in the S phase as compared to their wild-type counterparts (32% *vs.* 19%, and 31% *vs.* 13%, respectively, p<0.005; Fig. 2H). Moreover, in soft agar colony formation assays, both PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 cells formed significantly more colonies than parental PC3 and DU145 (p<0.005; Fig. 2I). In summary, *FRMD6* inhibited PC cell viability, proliferation, cell cycle progression, and anchorage-independent growth.

## FRMD6 inhibits 3D spheroid proliferation

Next, we investigated the effect of *FRMD6* knockout in 3D cell culture. PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 spheroids grew significantly faster than their corresponding controls (p<0.05; Fig. 3A, B and Supplementary Fig. S6A). We observed no differences in the number of spheroids formed for PC3 nor DU145 cells upon knockout of FRMD6 (28/28 wells; 100% for each cell line). After day 7, the faster growing PC3\_KO\_FRMD6 spheroids, but not DU145\_KO\_FRMD6, displayed an increasingly irregular morphology characterized by multiple cellular protrusions extending beyond the rounded spheroid core (Fig. 3C and Supplementary Fig. S6A). In addition, we found a significant increase in cell death and apoptosis for PC3\_KO\_FRMD6 spheroids (p<0.0001; Fig. 3C; Supplementary Fig. S6B, C), whereas cell death, but not apoptosis, was increased in DU145\_KO\_FRMD6 spheroids (p<0.0001; Supplementary Fig. S6D, E). This is likely attributable to hypoxia and nutrition depletion in the center of hyperproliferation spheroids. Furthermore, the altered morphology may reflect a more aggressive phenotype with disruption of cell-cell junctions and epithelial architecture consistent with previous reports [[6](#_ENREF_6)].

## FRMD6 inhibits tumor xenograft growth *in vivo*

For *in vivo* analysis, PC3\_KO\_FRMD6 cells and their parental and scrambled controls were subcutaneously injected into the flank of NMRI male nude mice (n=6 in each group; Fig. 3D). The tumor volume of PC3\_KO\_FRMD6 xenografts was significantly larger and tumors grew significantly faster than parental PC3 and PC3\_SCR xenografts (p<0.05; Fig. 3E, F). Due to substantial tumor burden, three mice with PC3\_KO\_FRMD6 xenografts were sacrificed after 24 days and another three after 32 days, whereas all PC3 and PC3\_SCR xenografts were sacrificed at the predefined endpoint of 45 days. Taken together, the 2D, 3D, and xenograft results highlight FRMD6 as a putative tumor suppressor gene in PC.

## FRMD6 loss activates c-MYC signaling

To elucidate how FRMD6 exerts its proposed tumor suppressor function, we conducted RNA-Seq and proteomic profiling (Supplementary Data 1+2, respectively) of parental PC3 and PC3\_KO\_FRMD6, which showed prominent phenotypic changes *in vitro* and *in vivo*. Gene Set Enrichment Analysis (GSEA) found that targets of the oncogenic c-MYC transcription factor (TF) were particularly enriched in the transcriptome of PC3\_KO\_FRMD6 (FDR<0.005; Fig. 4A, B). Concordant with this, Gene Ontology (GO) analysis of the top500 differentially expressed proteins revealed significant enrichment of c-MYC targets in PC3\_KO\_FRMD6 (Fig. 4C). Integrative analysis showed enrichment of multiple ribosomal, peptide biogenesis and mitochondrial gene sets in PC3\_KO\_FRMD6 (Fig. 4D), which is consistent with both c-MYC activation [[21](#_ENREF_21)] as well as the oncogenic phenotypes associated with *FRMD6* loss (Fig. 2D-F; 3A-C; Supplementary Fig. S6A). Moreover, we found that extracellular matrix (ECM) and multiple ECM binding components, such as collagen, fibronectin, integrin, and laminin gene sets, were significantly depleted in PC3\_KO\_FRMD6 (Fig. 4B, D). This is consistent with the irregular spheroid morphology observed in PC3\_KO\_FRMD6 spheroids (Fig. 3C) and regulation of cell-cell junctions critical for epithelial structure [[6](#_ENREF_6)]. Integrative analyses showed no evidence for enrichment of gene sets related to cell death or apoptosis, supporting the notion that the increased cell death and apoptosis rates observed for the 3D spheroids were likely attributed to nutrition/oxygen depletion.

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## Pyroxamide inhibits cell proliferation

To identify drugs that may inhibit the oncogenic phenotypes associated with *FRMD6* loss, we employed the Connectivity Map (CMap) [[22](#_ENREF_22)]. Querying CMap with a signature containing the top150 most upregulated transcripts in PC3\_KO\_FRMD6 (Supplementary Data 3), we obtained an inversely correlated connectivity score (CS) to c-MYC knockdown (CS: -99.95; Fig. 5A). To identify potential drugs, we ranked the ~3000 drugs in CMap according to CS and recognized several histone deacetylase inhibitors (HDACi), including pyroxamide, givinostat, panobinostat, and vorinostat among top candidates (Fig. 5A). We selected the HDAC1 inhibitor pyroxamide, based on its inversely correlated CS in PC3 and VCaP (-99.43 and -97.13, respectively), while minimizing the CS in non-PC cell lines (Fig. 5A). Treatment with pyroxamide resulted in a potent reduction of cell viability in a dose-dependent manner in PC3\_KO\_FRMD6 (IC-50 ≈6 µM; Supplementary Fig. S7). Concordantly, pyroxamide significantly inhibited PC3\_KO\_FRMD6 cell proliferation (p<0.005; absolute reduction in cell-index at 120h = 1.43; Fig. 5B), while only a small effect was observed in parental PC3 controls (p<0.05; absolute reduction in cell-index at 120h = 0.19; Fig. 5B). These results suggest that *FRMD6* deficient PC cells may be vulnerable to treatment with pyroxamide and provides pre-clinical proof-of-principle for drug repurposing experiments.

## FRMD6 activates Hippo signaling in PC cells

As FRMD6 has been proposed as an upstream activator of the Hippo signaling pathway [[7](#_ENREF_7)], we profiled the phospho-proteome in PC3\_KO\_FRMD6 and parental PC3. Hierarchical clustering separated both the proteome and phospho-proteome into two clusters according to *FRMD6* status (Supplementary Fig. S8). Querying the top250 differentially expressed phospho-peptides (Supplementary Data 4), we observed enrichment of GO terms related to mTORC1, heterochromatin, c-MYC/MAX, and Hippo/YAP signaling (Fig. 5C). Concordant with the literature, we focused on the Hippo pathway and investigated the abundance of protein and phospho-peptides for canonical Hippo members (Supplementary Fig. S9) [[23](#_ENREF_23)]. The bulk of Hippo core proteins (MST1/2, MOB1A, SAV1 and YAP) and most Hippo-associated members (e.g. KIBRA, MERLIN, PPP2CA, RASSF1, and AMOT) were significantly depleted upon *FRMD6* knockout (FDR<0.05; Fig. 5D, E). Likewise, the phosphorylation level of the canonical Hippo phospho-peptides (MOB1A, LATS1, and YAP) were significantly depleted at the indicated pSTY residues (FDR<0.05; Fig. 5F) [[7](#_ENREF_7), [8](#_ENREF_8)]. Consistent with Hippo-YAP dependent c-MYC activation, we observed a significant upregulation of several protein members of the AP-1 transcription factor complex, including JUN, JUNB, and FOSL2 (Supplementary Fig. S10) [[24](#_ENREF_24)]. These results suggest that FRMD6 is as a potent activator of Hippo signaling, and that *FRMD6* lossleads to disruption of the Hippo phosphorylation cascade and oncogenic transformation. To the best of our knowledge, this is the first report to associate FRMD6 with Hippo and c-MYC signaling in PC cell lines.

## *In vivo* knockout of *Frmd6* in the murine prostate

To investigate the function of FRMD6 in an androgen receptor (AR) positive cell line, we established LNCaP\_KO\_FRMD6 and observed a significant increase in cell viability compared to scrambled or parental controls (Fig. 6A; Supplementary Fig. S11A-C). To further characterize the endogenous role of *Frmd6* in a non-tumorigenic and AR positive *in vivo* environment, we injected Adeno-Associated Virus (AAV) particles containing sgRNAs for *Frmd6* and *Pten* (combined knockout) into the anterior prostatic lobe of Cas9 transgenic male mice (n=6). As controls (n=6), we used AAV particles with a sgRNA targeting only *Pten*, as orthotopic knockout of *Pten* is known to induce prostatic intraepithelial neoplasia (PIN) in mouse models and accelerate tumor formation [[25](#_ENREF_25), [26](#_ENREF_26)]. Anterior prostate tissue samples were analyzed 3 months post-transduction for indels generated by CRISPR/Cas9. TIDE analysis revealed indels in *Pten* in the controls, and in both *Frmd6* and *Pten* in the double-knockouts, confirming effective *in vivo* genomic editing in prostatic tissue (Fig. 6B). Furthermore, histopathological analysis revealed that *Frmd6/Pten* double-knockout promoted hyperproliferation and pre-malignant high-grade PIN (HG-PIN) lesions (Fig. 6C, D; lower), while *Pten* deficient specimens displayed a less aggressive phenotype with PIN lesions (Fig. 6C, D; upper). IHC staining for p-Akt showed increased activation of Akt in both controls (Fig. 6E; upper) and in double knockouts (Fig. 6E; lower), consistent with *Pten* loss in prostate epithelial cells. IHC staining for Frmd6 confirmed luminal Frmd6 expression in the wild-type mouse prostate (Fig. 6F, G) as well as loss of protein expression in areas with HG-PIN lesions in the double knockouts (Fig. 6H; lower). Using Ki-67 staining, we quantified the number of proliferating cells in PIN areas of *Pten* single-knockouts and in HG-PIN areas of *Frmd6/Pten* double-knockouts. There was a significantly higher number of proliferating (Ki-67+) cells in *Frmd6/Pten* double-knockout as compared to *Pten* single-knockout samples (p<0.05; Fig. 6I, J). These results indicate that *Frmd6* loss promotes pre-malignant prostate carcinogenesis and prostatic cell proliferation in prostatic tissue *in vivo*, further highlighting *Frmd6* as a novel tumor suppressor gene in PC.

**Discussion**

This study is the first to demonstrate FRMD6 tumor suppressor functions in PC. Specifically, functional studies in 2D, 3D, and xenografts showed that *FRMD6* loss increased PC cell viability, proliferation, cell cycle progression, anchorage-independent colony formation, spheroid growth, and subcutaneous xenograft tumor growth. FRMD6 also promoted development of premalignant HG-PIN in the murine prostate with concomitant *Pten* loss. Comprehensive transcriptome, proteome, and phospho-proteome profiling revealed activation of oncogenic c-MYC signaling and inactivation of the Hippo phosphorylation cascade. The present report is also the first to demonstrate significant downregulation and aberrant promoter hypermethylation of *FRMD6* in PC compared to NM prostate tissue samples from clinical patients, indicating an epigenetic silencing mechanism. Moreover, in two large PC patient cohorts, FRMD6 downregulation predicted post-operative BCR independently of routine clinicopathological parameters. This highlights the potential of FRMD6 as a novel prognostic biomarker candidate in PC and warrants further large-scale validation.

This is the first study to investigate the biological role of *FRMD6* in PC development and progression. Our findings that *FRMD6* knockout increased PC cell viability, proliferation, cell cycle progression, colony formation, and subcutaneous xenograft tumor growth are concordant with previous reports of tumor suppressor functions for FRMD6 in glioblastoma [[27](#_ENREF_27)] and hepatocellular carcinoma [[28](#_ENREF_28)] cell lines. Our results are also consistent with a study in breast cancer cell lines, where FRMD6 overexpression inhibited cell proliferation, colony formation and anchorage-independent growth *in vitro* as well as xenograft tumor growth [[29](#_ENREF_29)]. The irregular morphology observed for PC3 spheroids with *FRMD6* knockout is in agreement with a previous study, which found FRMD6 to be involved in maintaining epithelial structure through cell-cell junction complexes [[6](#_ENREF_6)]. The observation of increased epithelial markers and decreased mesenchymal markers in PC3\_KO\_FRMD6 may seem to conflict with the more aggressive phenotypes observed in these cells. However, the biology behind aggressiveness is complex, and cells may not adhere to strict epithelial/mesenchymal phenotypes. Indeed, recent reports show that cancer cells can metastasize in ways alternative to the traditional EMT paradigm, e.g. by detaching in clusters or existing in stable hybrid epithelial/mesenchymal [[30-32](#_ENREF_30)].

While previous studies [[27](#_ENREF_27), [29](#_ENREF_29)] demonstrated tumor suppressor function for FRMD6 in multiple cancer types, neither offered direct evidence for the effects being mediated through Hippo signaling. Our phospho-proteomics analyses showed that multiple Hippo kinases were dephosphorylated in response to *FRMD6* knockout. It has previously been reported that this causes deactivation of the Hippo phosphorylation cascade and thus activation of YAP/TAZ [[7](#_ENREF_7), [33](#_ENREF_33)]. We observed no difference in MST1/2 phosphorylation, which is consistent with previous studies demonstrating MST-independent, but LATS-dependent, activation of YAP/TAZ [[34](#_ENREF_34)].

We have searched the literature for a possible known link between Hippo and c-MYC signaling. We focused on the major signaling hub in the Hippo pathway, YAP/TAZ, which has no DNA-binding domains and thus relies on transcription factor (TF) partners, e.g. TEAD1-4 and KLF4, to initiate transcription of target genes [[33](#_ENREF_33), [35](#_ENREF_35)]. Previous work by Zanconato et al. [[24](#_ENREF_24)] showed that YAP/TAZ/TEAD-bound enhancers interact with the c–MYC promoter via chromatin looping in MDA-MB-231 cells. Our results are consistent with a model in which FRMD6 loss entails YAP/TAZ activation, and hence direct activation of c-MYC transcriptional expression. In addition, AP-1 promotes YAP/TAZ/TEAD-dependent gene expression and enhances YAP-mediated oncogenic growth [[24](#_ENREF_24)]. Thus, our results are also consistent with a model in which FRMD6 loss leads to increased c-MYC expression via activation of YAP/TAZ/TEAD/AP-1 interaction. In further agreement with this, we identified the HDAC1 inhibitor pyroxamide as a potent inhibitor of cell proliferation in *FRMD6* deficient PC cells. HDAC1 inhibitors are known to inhibit c-MYC by lysine 323 acetylation and interfere with chromatin remodelling in conjugation with HDAC1[[36-38](#_ENREF_36)], thus suggesting a possible mechanism for the vulnerability to pyroxamide in *FRMD6* deficient PC cells.

To investigate the biological role of FRMD6 in a non-tumorigenic environment, we utilized the Rosa26 transgenic mouse to co-target *Pten* and/or *Frmd6* [[39](#_ENREF_39)]. Loss of Pten is known to induce prostate gland enlargement and PIN lesions within a relatively short period of 12 weeks in Rosa26 mice [[25](#_ENREF_25)]. This model is clinically relevant since loss of PTEN is a recurrent genomic alteration in early stage PC patients [[40](#_ENREF_40)]. Our findings indicate that loss of Frmd6 may play a driver role in the early steps of PC development, by accelerating formation of HG-PIN lesions, which are considered to be precursors of malignant PC in mice [[41](#_ENREF_41)] and humans [[42](#_ENREF_42)]. We observe no development of either primary PC lesions or metastases in the Rosa26 model within 12-weeks. Further studies are warranted to determine whether *Frmd6/Pten* loss can drive the full transformation to malignant PC and/or metastatic progression after an extended period of time. To the best of our knowledge, this is the first study to show tumor suppressor function of endogenous *Frmd6* in the murine prostate.

In conclusion, we identified *FRMD6* as a novel tumor suppressor gene and prognostic biomarker candidate in PC. This was supported by clinical data from multiple PC patient sets, which showed gradual downregulation of FRMD6 expression from NM prostatic tissue, to primary PC tissue, and to advanced mCRPC samples as well as increased recurrence risk for patients with low FRMD6 expression. Furthermore, results from multiple *in vitro* and *in vivo* functional assays as well as multi-omics analyses showed that *FRMD6* has tumor suppressor functions in PC.

**Materials and methods**

Cell line and animal experiments are described in Supplementary Methods. Primers and oligos are listed in Supplementary Table S4, S5, and S6.

## Total RNA-Seq: Set#1 (discovery cohort) and isogenic PC cell lines

**RNA-Seq.** All samples used for RNA-Seq had an RNA integrity number (RIN) score >7. Whole transcriptome, strand-specific RNA-Seq libraries were prepared using Ribo-Zero Gold and ScriptSeq v2 kit (Epicentre) and paired-end sequencing on Illumina HiSeq 2000 (12–46 million reads/sample). Paired de-multiplexed FastQ files were generated using CASAVA software (Illumina) and reads were mapped to the human genome (hg19) using TopHat [[43](#_ENREF_43)] with the Bowtie aligner [[44](#_ENREF_44)]. HTSeq [[45](#_ENREF_45)] was used to summarize reads per gene of interest with the "union" overlap resolution mode. Fragments per kilobase of exon per million fragments mapped (FPKM) values were calculated using the Tuxedo suite [[46](#_ENREF_46)].

**Set#1**. A total of 55 PC and 18 NM prostate tissue samples (Supplementary Table S1) were collected at Department of Urology, Aarhus University Hospital, Denmark (2004-2016). Total RNA was isolated from fresh-frozen (tissue tek) and macro dissected prostate (cancer) tissue samples from radical prostatectomy (RP) specimens using the RNeasy Mini Kit (Qiagen) as previously described [[17](#_ENREF_17), [47](#_ENREF_47)].

**Set#2:** Genome-wide DNA methylome data were available from 21 PC and 21 NM macro-dissected RP specimens analyzed on the Infinium HumanMethylation450 (450K) BeadChip (Illumina) [[17](#_ENREF_17)]. Raw 450K data was processed using the ChAMP package (v1.2.0) [[48](#_ENREF_48), [49](#_ENREF_49)] in R [[50](#_ENREF_50)]. Each CpG site was assigned a β‐value ranging from 0 (unmethylated) to 1 (fully methylated).

**Set#3:** Genome-wide DNA methylome data (450K) and matched total RNA-Seq data were available from a distinct set of 22 PC and 12 NM laser capture micro‐dissected RP prostate tissue samples, as previously described [[15](#_ENREF_15), [16](#_ENREF_16)].

## External PC patient datasets

**TCGA/PRAD** [[19](#_ENREF_19)]: RNA-Seq data (FPKM values) and DNA methylation data (Beta values, Illumina 450K array) were downloaded from the TCGA data portal [[18](#_ENREF_18), [19](#_ENREF_19)], as previously [[51](#_ENREF_51)]. FRMD6 expression and DNA methylation data was available for 479 PC and 29 adjacent normal (AN) specimens.

**Long et al.** [[14](#_ENREF_14)]: RNA-Seq data (FPKM values) for 106 RP patients was downloaded from the Gene Expression Omnibus (GEO) database (accession number GSE54460). For biochemical recurrence (BCR) free survival analysis, we excluded 10 patients who suffered BCR within one month from RP (Supplementary Table S3) and 8 patients with missing clinical data.

**Taylor et al.** [[12](#_ENREF_12)]: Affymetrix human exon 1.0 ST array data and clinical data [[20](#_ENREF_20)] was downloaded for 150 PC and 29 AN tissue specimens from GEO (accession number GSE21036). Clinical follow-up and FRMD6 expression data were available for 126 PC patients (Supplementary Table S3).

**Grasso et al.** [[13](#_ENREF_13)]: Whole Human Genome Microarray and Whole Human Genome Oligo Microarray data from 28 NM, 59 PC, and 35 mCRPC tissue specimens was downloaded from the GEO database (accession number GSE35988). FRMD6 expression data was available for 22 NM, 57 PC, and 29 mCRPC tissue specimens.

## Bioinformatics and statistical analyses

Statistical analyseswere performed using R [[50](#_ENREF_50)] or STATA v. 15 (StataCorp). Differential expression analysis was performed using EdgeR [[52](#_ENREF_52)] with maximum complex dispersion for each gene. Differential expression in external datasets was assessed by the Wilcoxon rank-sum test. Correlations between expression and methylation levels were analyzed using Spearman’s test. Differences in FRMD6 immunoreactivity scores and cell cycle distribution were assessed using the chi2 test. For functional studies in cell culture, Student’s t-test (two sided) was used to evaluate differences between treated and controls, unless stated otherwise.

BCR-free survival was assessed by Kaplan-Meier and uni-/multivariate Cox regression analyses. For multivariate testing, all clinicopathological parameters significant in univariate analysis were included. Predictive accuracy was estimated using Harrell’s C-index [[53](#_ENREF_53)]. For Kaplan-Meier analysis, FRMD6 expression was dichotomized based on receiver operating characteristics (ROC) curve analysis (BCR status at 36 months). Where applicable, correction for multiple testing (FDR, false discovery rate) was performed according to Benjamini-Hochberg [[54](#_ENREF_54)]. FDR and P-values <0.05 were considered significant.

## Study Approval

This study was approved by The Central Denmark Region Committee on Health Research Ethics (2000-0299) and notification was given to the Danish Data Protection Agency (No. 2013-41-2041). Written informed consent was obtained from all patients. All animal experiments were approved by the Danish Animal Experiments Inspectorate (permission 2013-15-2934-00901, C2 (Xenografts) and 2016-15-0201-01083 (Orthotopic knockout of Frmd6 in Rosa26 mice) and housed according to Danish legislation and the Directive 2010/63/EU on the protection of animals used for scientific purposes.

**Author contributions**. Conception and design: JHJ, SHS, KDS. Development of methodology: JHJ, SHS, CCB, MT, JVO. Acquisition of data JHJ, SHS, MR, SH, MN, MT, FDH, MB, MEJ. Analysis and interpretation of data: JHJ, SHS, MEJ, MT, CCB, BU, KDS. Writing of the manuscript: JHJ, SHS, KDS. Revision and approval of the final manuscript: All authors. Study supervision: KDS, ZKJ, RE, JVO.

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# Figure Legends:

Figure 1: FRMD6 in clinical samples. (A) FRMD6 expression (RNA-Seq) in 18 NM and 55 PC tissue samples. P-values from EdgeR. (B) FRMD6 expression (RNA-Seq) in NM (PrEC) and isogenic PC cell lines (DU145 vs. DU145-MN1, PC3 vs. PC-3M). (C-F) FRMD6 transcriptional expression in tissue samples from (C) 29 NM and 479 PC (TCGA), (D), 29 NM and 150 PC (Taylor et al.), (E) 22 NM, 57 PC, and 20 mCRPC (Grasso et al.), (F) 106 PC (55 BCR+, 51 BCR-, Long et al.) P-values from Wilcoxon test (C-F). (G) DNA methylation (450K, in-house data) of 5 CpG sites in FRMD6 promoter in 43 PC and 33 NM tissue samples. FRMD6 transcription start site marked by arrow. FDR from ChAMP analysis. (H) ROC curve analyses of the mean DNA methylation of the 5 CpG sites in (G). P-values from Wilcoxon test. (I) DNA methylation (450K) of 5 CpG sites in the FRMD6 promotor region in 497 PC vs. 29 NM tissue samples from TCGA. The arrow marks the FRMD6 transcription start site. P-values from Wilcoxon test. (J) ROC curve analysis of the mean DNA methylation of the 5 CpG sites in (i), P-value from Wilcoxon test. (K) FRMD6 expression (RNA-Seq) vs. promotor DNA methylation (450K) in 22 PC and 12 N; tissue samples (in-house data). Rho and p-value from Spearman correlation analysis. (L) FRMD6 expression (RNA-Seq) vs. promotor DNA methylation (450K) in 497 PC and 29 NM tissue samples (TCGA) Rho and P-value from Spearman correlation analysis. (M-N) Kaplan-Meier analysis if time to BCR for patients stratified by FRMD6 expression in the (M) Taylor, and (N) Long cohorts. P-values from log-rank test. AUC: Area under the curve, \*p>0.01, \*\*p>0.001, \*\*\*p>0.0001.

Figure 2: Functional studies in isogenic PC3, PC-3M, DU145, and DU145-MN1 cell lines. (A) Viability of PC3 and DU145 cells with/without FRMD6 overexpression/mock. Results are presented relative to the corresponding WT and SCR. (B) Proliferation of PC-3M and (C) DU145-MN1 cells with/without transient FRMD6 overexpression. Cell index was recorded every 15 min for 96h by xCELLigence. (D) Viability of PC3 and DU145 cells with/without FRMD6 KO or SCR. Results are presented relative to the corresponding WT. (E) Proliferation of PC3 and (F) DU145 cells with/without FRMD6 KO. Cell index was recorded every 15 minutes for 96h by xCELLigence. (G) Viability of PC3\_KO\_FRMD6 and DU145\_KO\_FRMD6 cells with/without transient FRMD6 rescue/mock. Results are presented relative to the corresponding KO or mock. (H) Cell cycle analysis of PC3 and DU145 with/without FRMD6 KO. The percentage of cells on G1, S, or G2 phase are presented. (I) Anchorage-independent colony formation for PC3 and DU145 cells with/without FRMD6 KO/SCR. Colonies were counter under light microscope after 21 days. All viability readouts were performed 72h post-seeding using AlamarBlue. Significant p-values (two-sided Student´s t-test) are marked by \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005, or \*\*\*\*p<0.0001 for all figures except (H) (Chi-square test). Results are presented as the ±SD of a minimum of three replicates. WT: wild-type, SCR: scrambled control, KO\_ knockout.

Figure 3. FRMD6 inhibits 3D spheroid and xenograft tumor growth. (A-B) Spheroid size (mm2) at indicated time points for (A) PC3 and (B) DU145 cells with/without FRMD6 KO/SCR. Results are the mean ±SD of a minimum of 15 replicates. (C) Representative pictures of 3D spheroid size/morphology, necrosis, and apoptosis at indicated time points. An untreated (UT), propidium iodide (PI), and caspase (Casp) readout was performed to measure the level of necrosis and apoptosis, respectively, in the indicated PC3 cell line. Scale bars: 500 um. (D) Indicated cells were embedded in Matrigel were subcutaneously injected into the flank of 6 immunocompromised (nude) NMRI mice. (E) Subcutaneous tumor growth curve of PC3 (black), PC3\_SCR (grey), and PC3\_KO\_FRMD6 (red) cells in NMRI male nude mice (n=6 per group). Mice were sacrificed at the time points marked by dotted vertical lines. (F) Dot plot of the maximum tumor volume for each xenograft. Results are the mean ±SD of six replicates. Significant p-values (two-sided Student´s t-test) are marked by \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005, or \*\*\*\*p<0.0001. WT: wild-type, SCR: scrambled control, KO: knockout.

Figure 4. Pathway analysis of PC3\_KO\_FRMD6 vs. parental PC3 based on RNA-Seq data. Gene Set Enrichment Analysis (GSEA) using (A) ´Hallmark´ and ´C6´ collection for PC3\_KO\_FRMD6 (triangles) vs. PC3 (squares). The normalized enrichment score (NES) of significantly depleted or enriched gene sets (FDR<0.005) are colored according to FDR q-value. (B) Log2 transformed FPKM values of selected differentially expressed genes. (C) Pathway analysis (Enrich) of top500 most abundant proteins in PC3\_KO\_FRMD6 vs. parental PC3 cells. Enriched transcription factors (TFs) and GO/Pathways are presented according to combined Z-score. (D) GSEA using the ´C5\_Gene Ontology´ collection (FDR<0.002) for PC3\_KO\_FRMD6 (triangles) vs. PC3 (squares). The normalized enrichment score (NES) of significantly depleted or enriched gene sets (FDR<0.002) are colored according to FDR q-value. All analyses were corrected for multiple testing. GO: Gene Ontology.

Figure 5. Drug repurposing experiments and phospho-proteomics. (A) Connectivity scores of c-MYC knockdown and several HDAC inhibitors, including pyroxamide, identified by querying top150 upregulated genes in PC3\_KO\_FRMD6 vs. parental PC3 to Connectivity Map. (B) Cell proliferation of PC3 and PC3\_KO\_FRMD6 cells treated with pyroxamide (6 uM). Pyroxamide was added after 48h and readouts were performed every 12h using xCELLigence for 72h. Results are presented as ±SD of a minimum of three replicates. (C) Pathway analysis (Enrichr) of top250 most abundant phospho-peptides in PC3\_KO\_FRMD6 vs. parental PC3. Enriched transcription factors (TFs) and GO/Pathways are presented according to combined Z-score and adjusted for multiple testing. (D) Proteomics analysis of core Hippo kinase enzymes and (E) other Hippo regulatory proteins in PC3\_KO\_FRMD6 vs. parental PC3. (F) The phosphorylation level of indicated serine (S), threonine (T), or tyrosine (Y) residues for significantly deregulated Hippo proteins are shown. Significant p-values (two-sided Student´s t-test) are marked by \*p<0.05 or \*\*p<0.005.

Figure 6. Orthotropic gene editing in transgenic Rosa26-LSL-Cas9-eGFP mice. (A) Viability of LNCaP cells with/without FRMD6 KO or SCR control. (B) TIDE-analysis of Pten and Pten/Frmd6 using DNA isolated from bulk prostate tissue samples. (C) H&E staining of Pten (n=6) and Pten/Frmd6 (n=6) deficient histological sections (4 um) of the anterior prostate lobe at 3 months after virus delivery. Scale bars: 100um. (D) Same as C) but with scale bars: 20 um. (E) IHC staining of p-AKT (brown) for representative areas with Pten KO or Pten/Frmd6 KO. Scale bars: 20um. (F) Representative IHC of Frmd6 in the anterior lobe of wild-type ROSA26 mice. Scale bars: 100um. (G) Close-up of the dashed area in F) with scale bars: 20um. A strong luminal Frmd6 straining is observed and confirms endogenous Frmd6 expression in the murine prostate. (H) IHC staining Frmd6 (red) for representative areas with Pten KO or Pten/Frmd6 KO. Arrows highlight representative positive cells. Scale bars: 20um. (I) Ki-67 staining of proliferating cells in representative PIN areas. Scale bars: 20um. (J) Quantification of Ki-67 positive cells from I). Data is presented as ±SD of 30 representative fields. Significant p-values (two-sided student´s t-test) are marked by \*p>0.05, \*\*p>0.005m \*\*\*p>0.0005. \*N/A: not available. H&E: Hematoxylin and eosin, SCR: Scrambled, KO: knockout, PX: Pten + none, PF: Pten + Frmd6.

# Table Legend:

Table 1: The prognostic potential of FRMD6 expression (analyzed as a continuous variable) in the Taylor (top) and the Long (bottom) cohorts. Univariate and multivariate Cox regression analyses of post-operative biochemical recurrence (BCR)-free survival. C-index1= Harrell’s C-index for model including FRMD6. C-index2= Harrell’s C-index for model excluding FRMD6. N/A = Not available. Significant p-values are highlighted in bold.