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Supplemental information

Seed-mediated RNA interference of androgen signaling and survival networks induces cell death in prostate cancer cells

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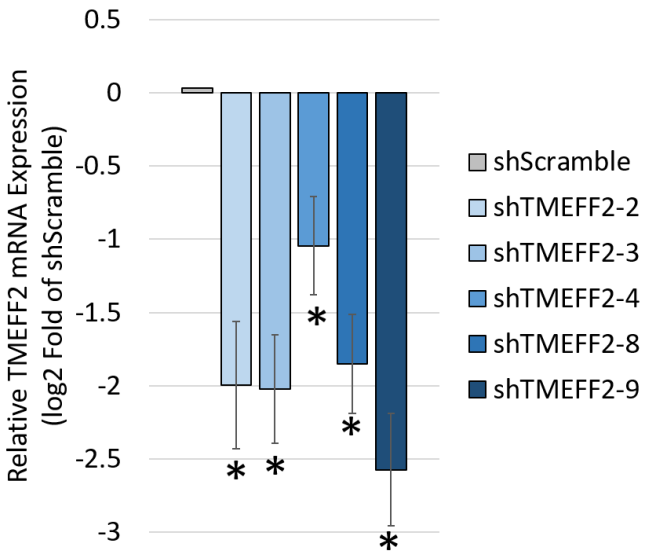

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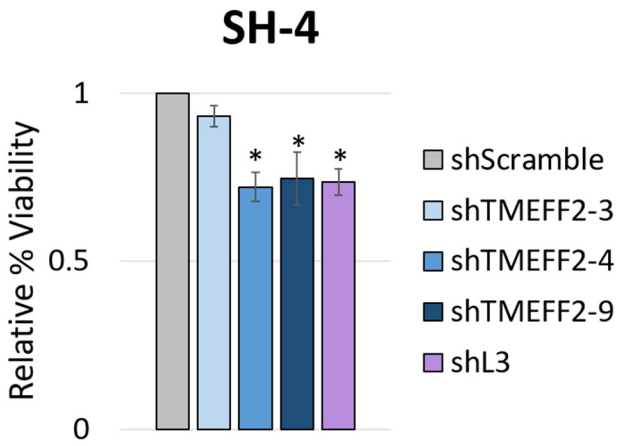


Figure S1. (A)TMEFF2 knockdown in response to TMEFF2-targeted shRNA (B) and effect on viability of melanoma cell line. (A) TMEFF2 mRNA expression in LNCaP cells expressing shTMEFF2-2, shTMEFF2-3, shTMEFF2-4, shTMEFF2-8 or shTMEFF2-9 relative to cells expressing shScramble control. RNA was extracted 72 hours after transductions, and mRNA expression was determined by RT-qPCR. N=3, error bars ±SD, * p<.05 determined by t-test. **(B)** Viability of melanoma (SH-4) cell line transduced with plasmids expressing TMEFF2-targeted (shTMEFF2-3, -4, -9) and CD95L targeted shRNAs (shL3) or shScramble control. Viability was determined by trypan blue and is presented as percent viability relative to shScramble. N=3, error bars ±SD, * p<.05 determined by t-test.

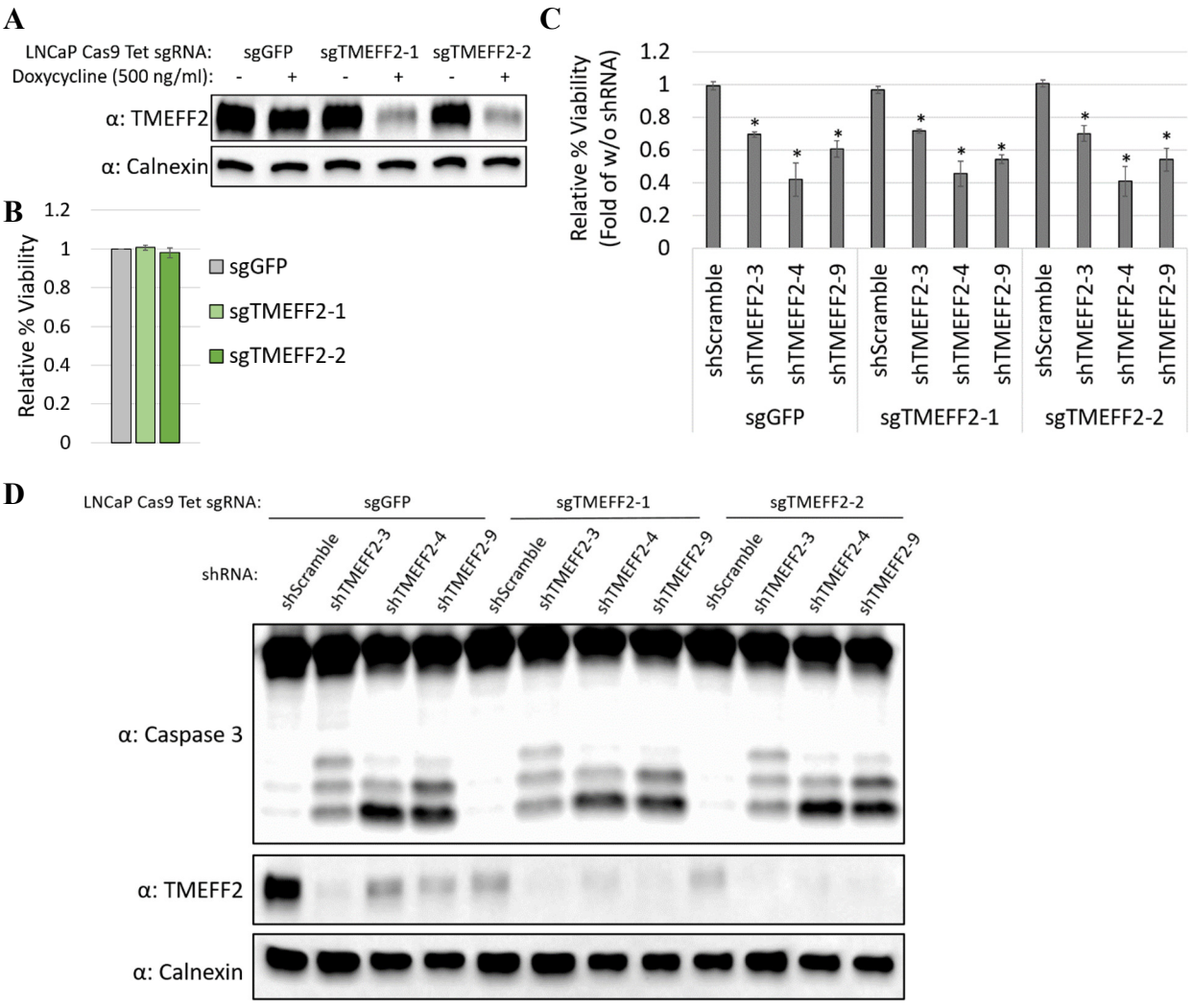


Figure S2. Loss of viability in response to TMEFF2-targeted shRNA is independent of TMEFF2 protein levels. (**A**) Western blot analysis showing doxycycline-induced TMEFF2 knockdown in LNCaP Cas9 cells expressing doxycycline-inducible TMEFF2-targeted sgRNAs (sgTMEFF2-1 and sgTMEFF2-2) after cells were grown for 10 days in the presence and absence of 500 ng/ml doxycycline. Doxycycline-inducible GFP targeted sgRNA (sgGFP) served as a negative control. Calnexin was used as a loading control. (**B**) Percent viability of LNCaP Cas9 sgTMEFF2-1 and sgTMEFF2-2 cells lines relative to LNCaP Cas9 sgGFP cell line. Viability determined by trypan blue after cells were grown in 500 ng/ml doxycycline for 10 days to induce sgRNA expression. N=3, error bars ±SD. (**C**) Relative percent viability and (**D**) western blot analysis showing TMEFF2 expression and caspase 3 cleavage in lysates from LNCaP Cas9 sgGFP, sgTMEFF2-1 and sgTMEFF2-2 cell lines grown in 500 ng/ml dox and subsequently transduced with plasmids expressing TMEFF2-targeted shRNAs or shScramble control. Viability was measured by trypan blue 96 hours after shRNA transductions. Viability is presented as percent viability relative to the viability of cells without shRNA expression. N=3, error bars ±SD, * $p<.05$ determined by t-test. Lysates were also obtained 96 hours after shRNA transductions, and calnexin was used as loading control.

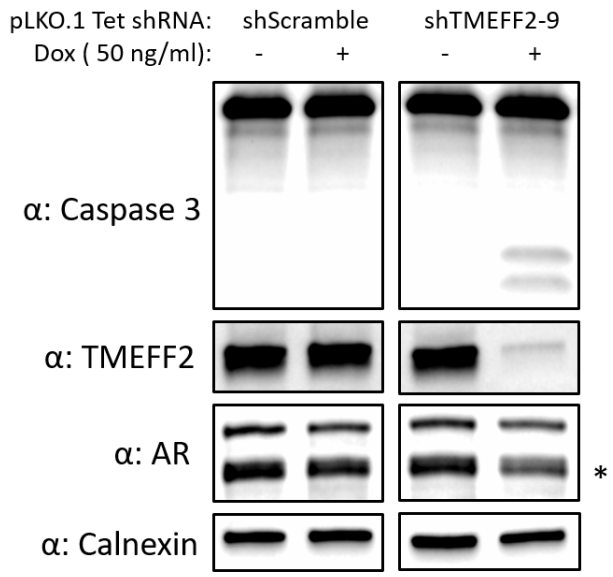


Figure S3. Doxycycline inducible TMEFF2-targeted shRNA expression induces caspase 3 cleavage and reduces AR protein expression in 22Rv1 cells. Western blot analysis of Caspase 3 cleavage, TMEFF2 and AR protein (* AR-V7) expression in lysates from 22Rv1 shScramble and shTMEFF2-9 cell lines grown in the presence and absence of 50 ng/ml doxycycline for 5 days to induce shRNA expression. Calnexin was used as loading control.

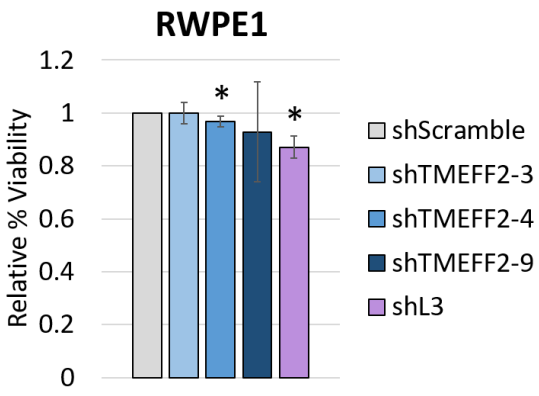


Figure S4. TMEFF2-targeted shRNAs and shL3 have minimal effect on normal prostate cell viability. Percent viability of RWPE1 cells expressing shTMEFF2-3, shTMEFF2-4, shTMEFF2-9 or shL3 relative to the viability of cells expressing shScramble control shRNA. Viability was measured by trypan blue 120 hours after transductions. N=3, error bars ±SD, * $p<.05$ determined by t-test.

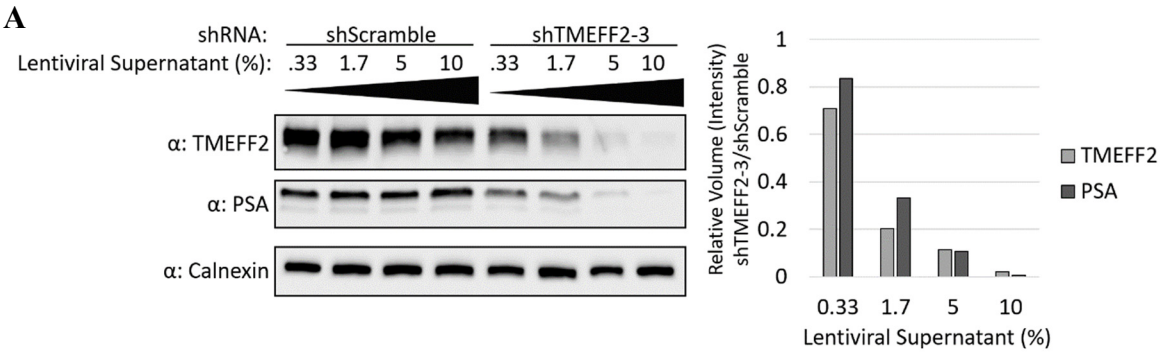


Figure S5. Low TMEFF2-targeted shRNA expression reduces PSA protein levels. Western blot analysis showing PSA and TMEFF2 protein levels in response to increasing dose of lentiviral particles containing plasmids expressing shTMEFF2-3 and shScramble shRNA in LNCaP cells, four days after transduction. Dose is presented as percentage of lentiviral supernatant in transduction media. See right panel for graphical representation of Calnexin normalized PSA and TMEFF2 levels in response to shTMEFF2-3 expression relative to shScramble control at each dose. Band intensity was quantified using Biorad Image Lab.

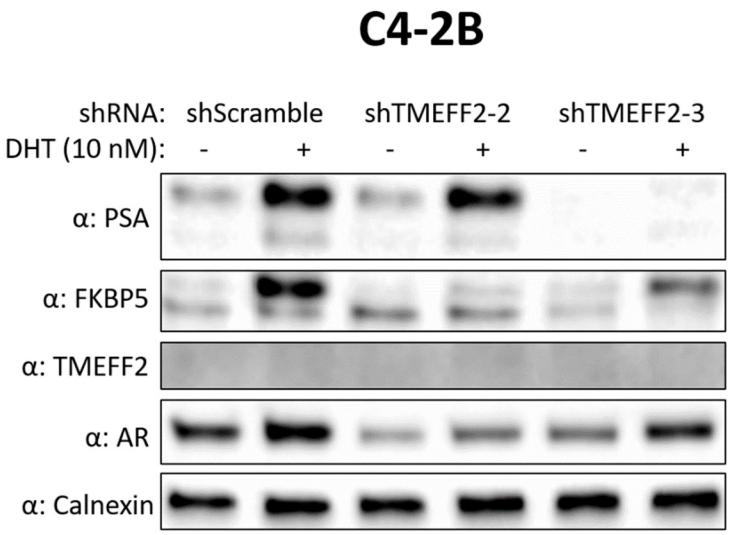


Figure S6. TMEFF2-targeted shRNAs reduce androgen responsive protein levels. TMEFF2, AR and androgen responsive protein expression in lysates from C4-2B cells expressing shTMEFF2-2, shTMEFF2-3 or shScramble control shRNAs and grown in the presence and absence of 10 nM DHT. Calnexin was used as loading control for all western blots.

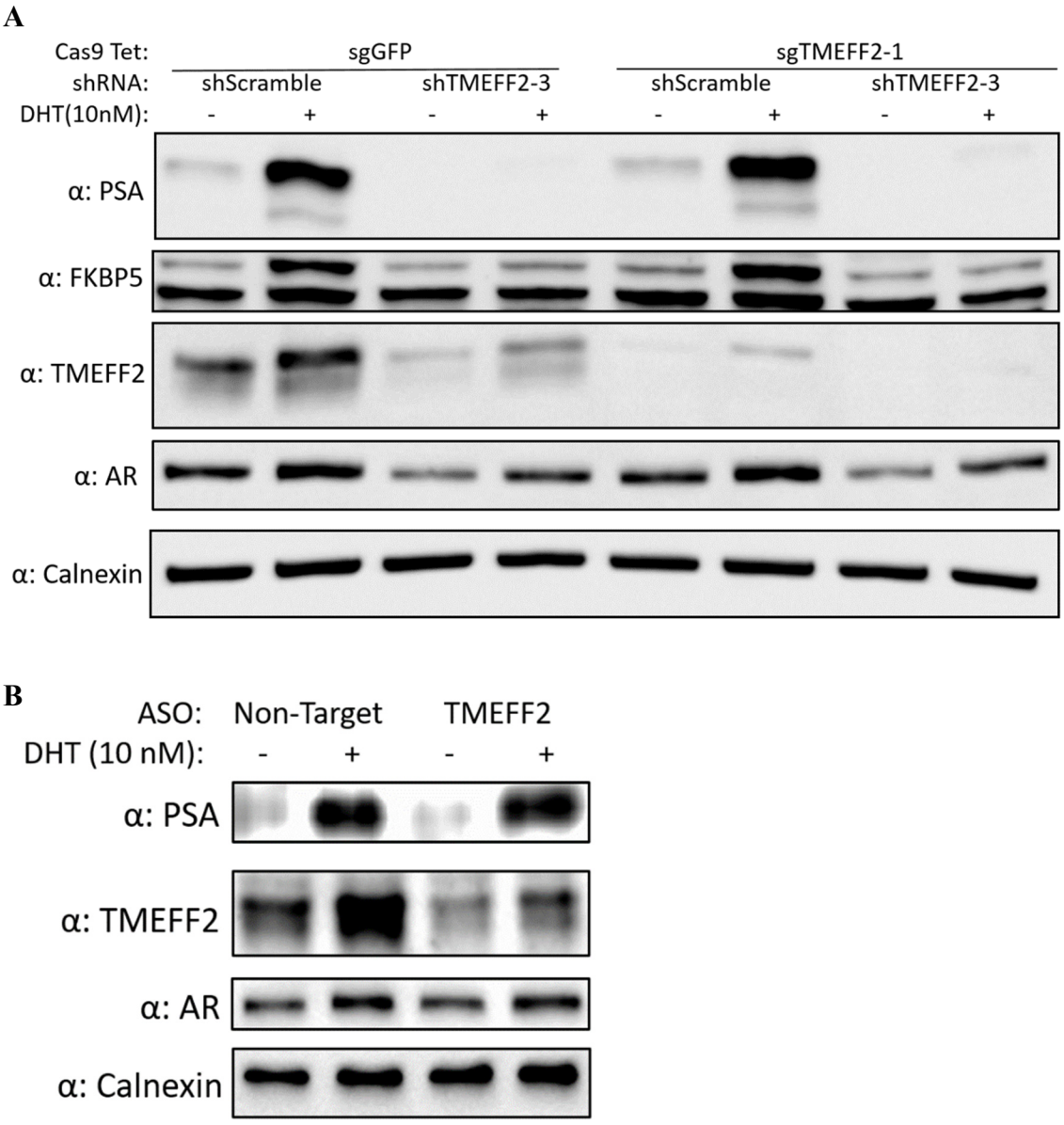
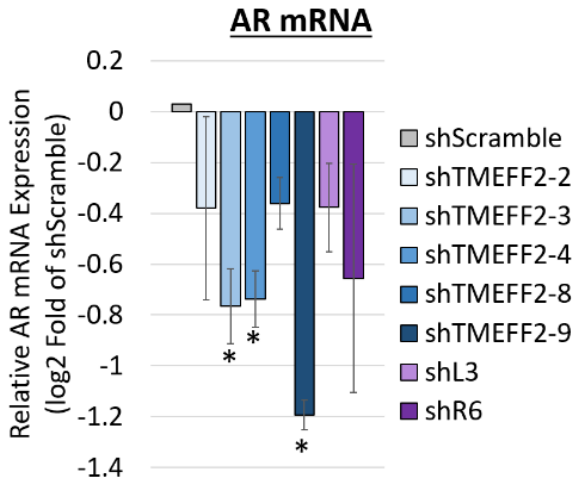


Figure S7. Inhibition of androgen signaling in response to TMEFF2-targeted shRNA is independent of TMEFF2 protein levels. (**A**) Western blot analysis showing TMEFF2, AR, PSA and FKBP5 protein levels. Lysates were from LNCaP Cas9 sgGFP and sgTMEFF2-1 cell lines, grown for 2 weeks in 500 ng/ml doxycycline to induce TMEFF2 knockdown, followed by 2 weeks in the absence of doxycycline, then transduced with plasmids expressing shScramble or shTMEFF2-3 shRNAs, and grown in the presence or absence of 10 nM DHT. (**B**) Western blot analysis showing PSA, TMEFF2 and AR protein levels in lysates from LNCaP cells transfected with Non-Target or a pool of TMEFF2 targeting ASO's, and grown in the presence and absence of DHT. Calnexin was used as a loading control for all western blots.

A



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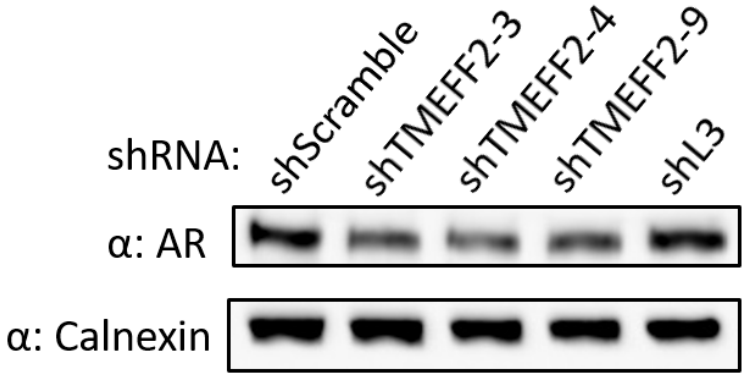
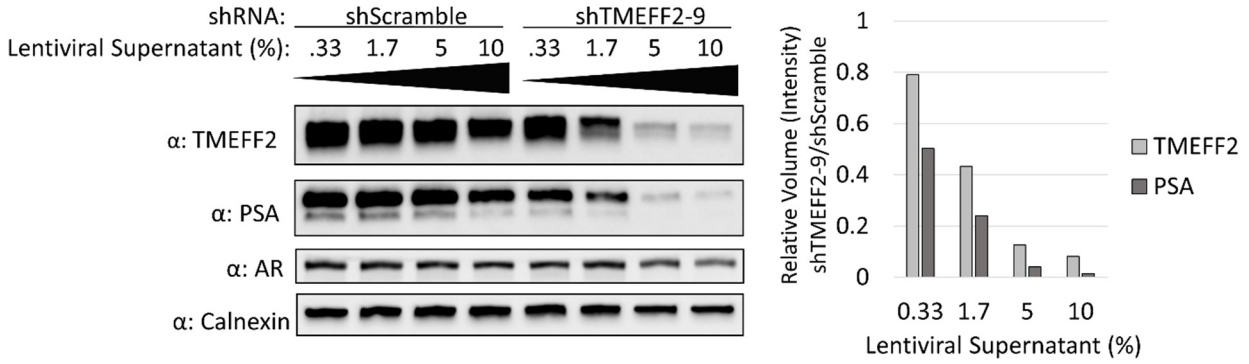


Figure S8. TMEFF2-targeted shRNAs reduce AR protein levels, while shL3 does not. (**A**) AR mRNA expression in LNCaP cells expressing TMEFF2-targeted shRNAs, shL3 or shR6 relative to cells expressing shScramble control. RNA was extracted 72 hours after transductions, and mRNA expression was determined by RT qPCR. RPL8, RPL38, PSMA1 and PPP2CA were the housekeeping genes used for normalization. N=3, error bars ±SD, * $p<.05$ determined by t-test. (**B**) Western blot analysis showing AR protein expression in lysates obtained from LNCaP cells expressing shTMEFF2-3, shTMEFF2-4, shTMEFF2-9, shL3 or shScramble control shRNA. Lysates were obtained 96 hours after transduction. Calnexin was used as loading control.

A



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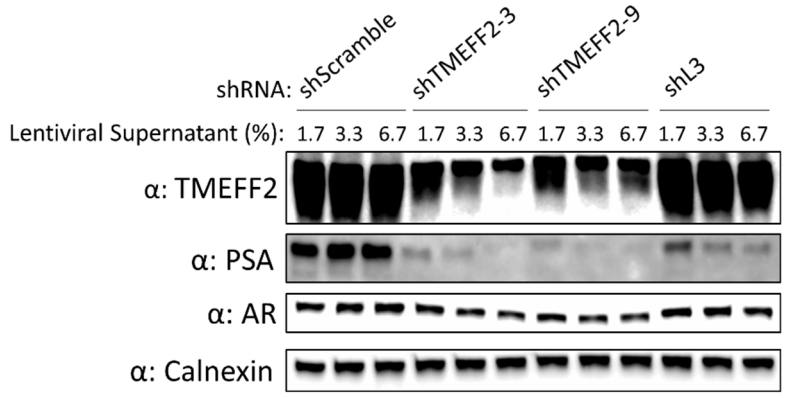


Figure S9. Low TMEFF2-targeted shRNA expression can reduce AR signaling targets independently of their effect on AR protein levels. Western blot analyses showing PSA, TMEFF2 and AR protein levels in response to increasing dose of lentiviral particles containing plasmids expressing shTMEFF2-9 and shScramble shRNA (**A**) and shTMEFF2-3, shTMEFF2-9 and shL3 in LNCaP cells (**B**) four days after transduction. Dose is presented as percentage of lentiviral supernatant in transduction media. See right panel for graphical representation of Calnexin normalized PSA and TMEFF2 levels in response to shTMEFF2-9 expression relative to shScramble control at each dose. Band intensity was quantified using Biorad Image Lab.

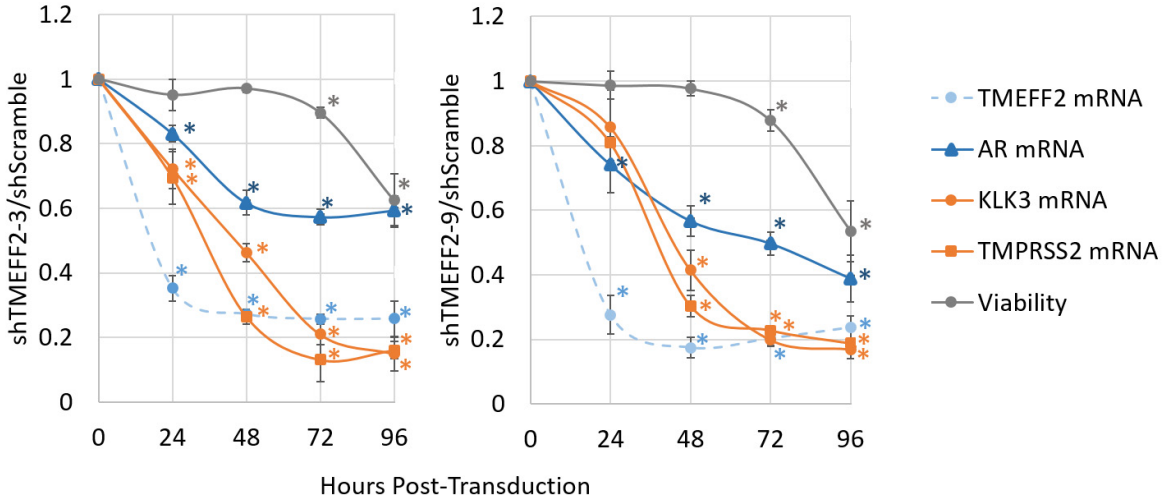
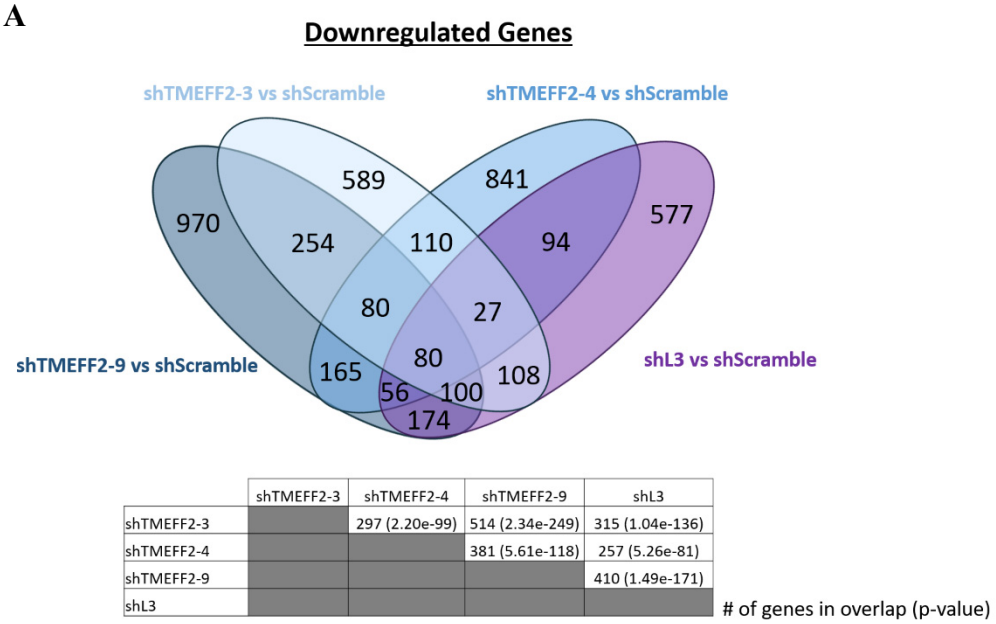


Figure S10. Reduction in AR and androgen responsive genes occur prior to loss of viability in response to TMEFF2-targeted shRNAs. Time course analysis of TMEFF2, KLK3, TMPRSS2 and AR mRNA expression and viability of LNCaP cells expressing shTMEFF2-3 (left panel) or shTMEFF2-9 (right panel) relative to cells expressing shScramble. Viability was determined by trypan blue, and mRNA expression was determined by RT qPCR. RPL8, RPL38, PSMA1 and PPP2CA were the housekeeping genes used for normalization. N=3, error bars ±SD, * $p<.05$ determined by t-test (for H_0: viability or expression = 1).

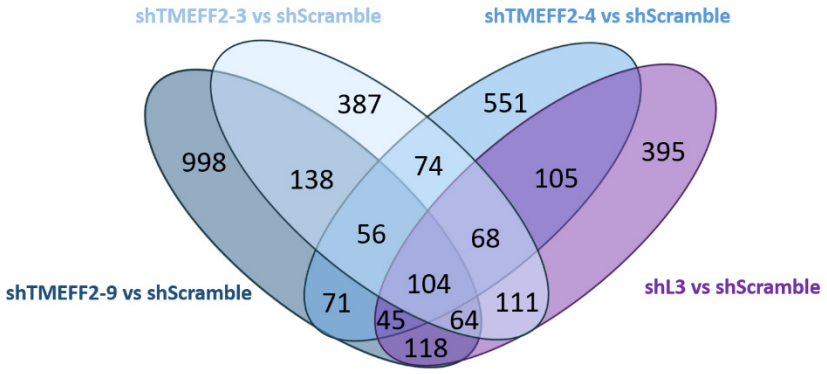
A



	shTMEFF2-3	shTMEFF2-4	shTMEFF2-9	shL3
shTMEFF2-3		297 (2.20e-99)	514 (2.34e-249)	315 (1.04e-136)
shTMEFF2-4			381 (5.61e-118)	257 (5.26e-81)
shTMEFF2-9				410 (1.49e-171)
shL3				

# of genes in overlap (p-value)

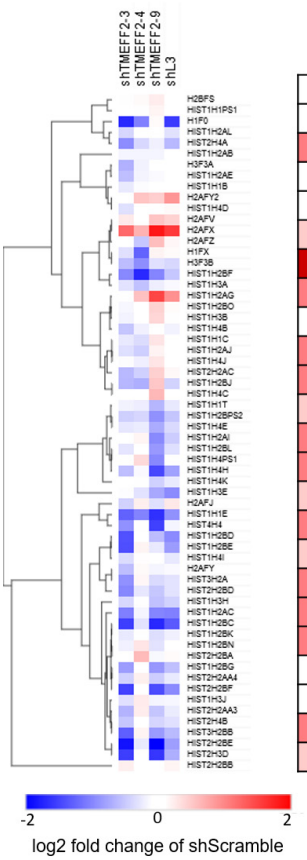
B



	shTMEFF2-3	shTMEFF2-4	shTMEFF2-9	shL3
shTMEFF2-3		302 (1.02e-181)	362 (3.21e-187)	347 (1.38e-245)
shTMEFF2-4			276 (6.12e-99)	322 (1.49e-203)
shTMEFF2-9				331 (4.60e-155)
shL3				

# of genes in overlap (p-value)

Figure S11. shTMEFF2-3, shTMEFF2-4, shTMEFF2-9 and shL3 expressing LNCaP cells exhibit overlaps in DEGs. Venn diagrams show the number of genes significantly downregulated (**A**) and upregulated (**B**) for each comparison from RNA-seq analysis of LNCaP cells expressing designated shRNAs. Tables show the number of overlapping DEGs and p-values indicating the significance of overlaps for pairwise comparisons of each shRNA based on a hypergeometric distribution.



Histone Genes Significantly Downregulated			
shTMEFF2-3	shTMEFF2-4	shTMEFF2-9	shL3
H1F0	H1F0	HIST1H1E	H1F0
H2AFY	H1FX	HIST1H2AC	HIST1H1E
H3F3A	H2AFZ	HIST1H2AI	HIST1H2AC
H3F3B	H3F3B	HIST1H2BC	HIST1H2BC
HIST1H1E	HIST1H1E	HIST1H2BD	HIST1H2BD
HIST1H2AC	HIST1H2BF	HIST1H2BF	HIST1H2BE
HIST1H2AE	HIST1H2BJ	HIST1H2BG	HIST1H2BG
HIST1H2BC		HIST1H2BL	HIST1H3E
HIST1H2BD		HIST1H3E	HIST1H4H
HIST1H2BE		HIST1H3H	HIST2H2BE
HIST1H2BF		HIST1H4H	HIST2H2BF
HIST1H2BG		HIST1H4PS1	HIST2H3D
HIST1H2BJ		HIST2H2BD	HIST3H2BB
HIST1H4H		HIST2H2BE	
HIST2H2BD		HIST2H2BF	
HIST2H2BE		HIST2H3D	
HIST2H2BF		HIST3H2BB	
HIST2H3D		HIST4H4	
HIST2H4A			
HIST3H2A			
HIST3H2BB			
HIST4H4			

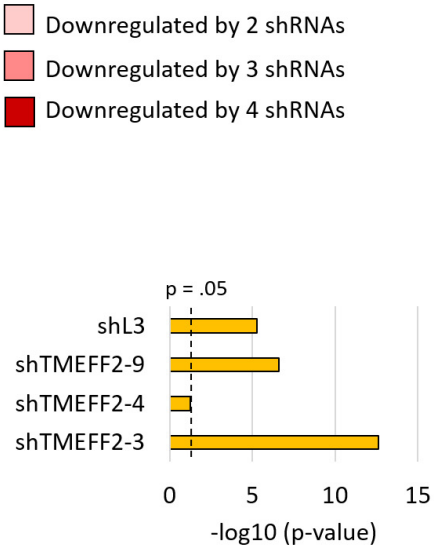


Figure S12. shTMEFF2-3, shTMEFF2-4, shTMEFF2-9 and shL3 downregulate histone mRNAs. Heatmap (Left panel) shows the expression of histone genes in LNCaP cells expressing designated shRNAs relative to LNCaP cells expressing shScramble shRNA according to RNA-seq analysis. Table shows histone genes significantly downregulated by each shRNA relative to LNCaP cells expressing shScramble shRNA. Commonly downregulated histone genes are highlighted in red. Bar graph shows –log10 p-value for enrichment of histone genes among significantly downregulated genes by each shRNA relative to shScramble expressing LNCaP cells. –log10 p-values are based on hypergeometric distribution.

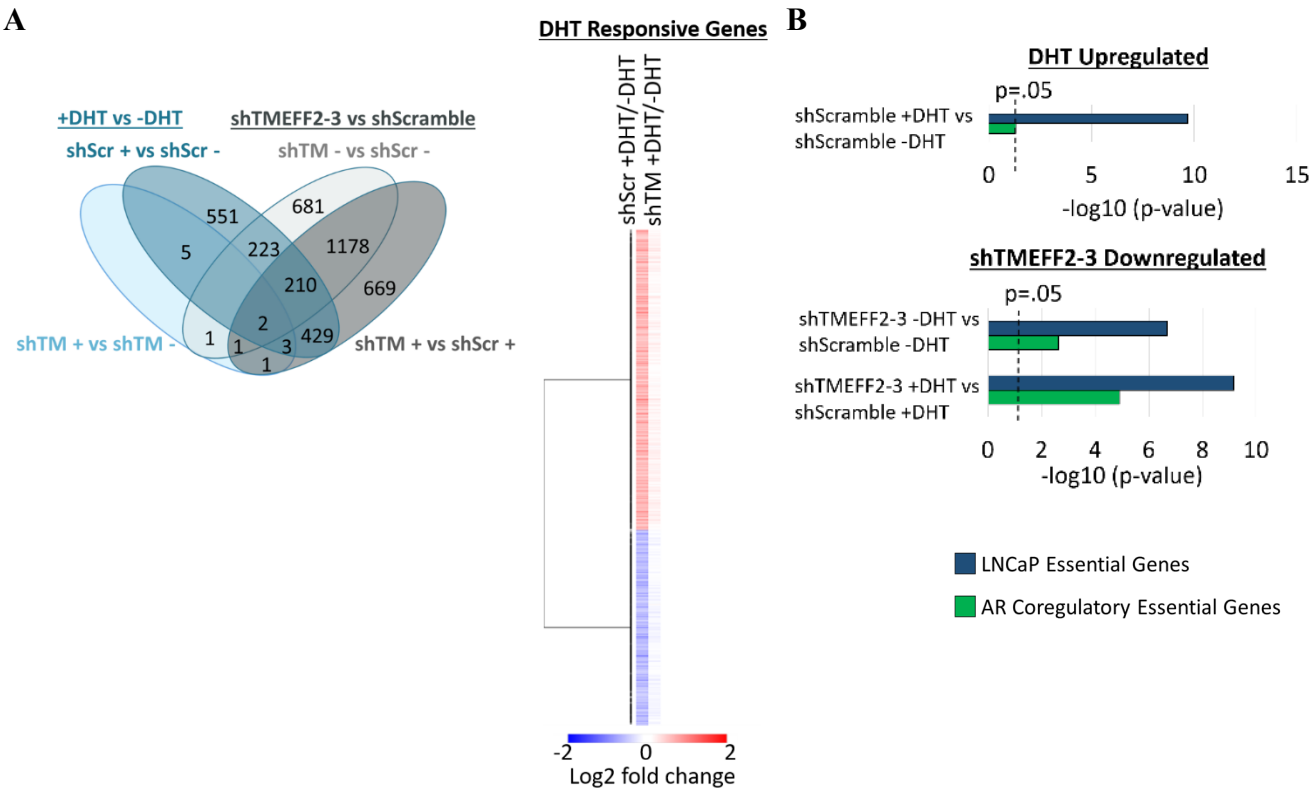
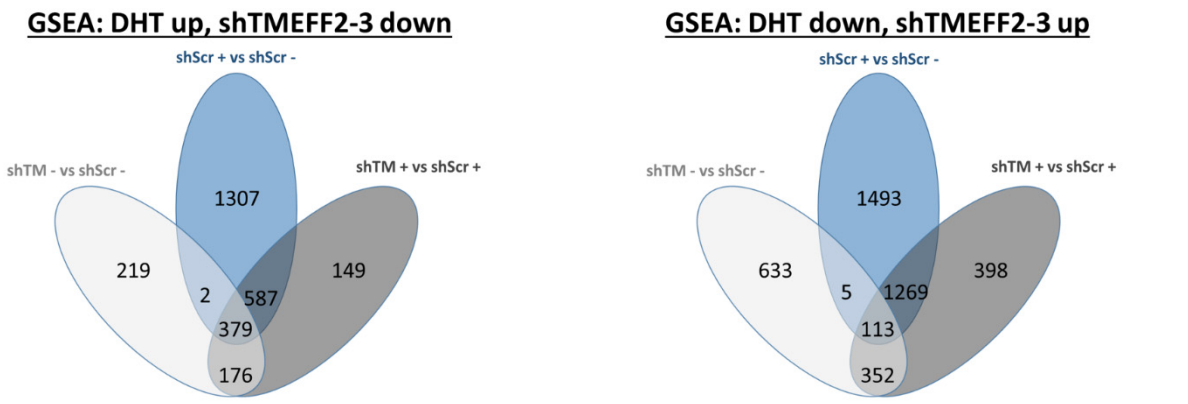


Figure S13. shTMEFF2-3 inhibits global androgen-induced transcriptional response. (**A**) Venn diagram shows the number of significantly differentially expressed genes (DEGs) between each comparison (shTM: shTMEFF2-3; Scr: shScramble; -: - DHT; +: + DHT) from RNA-seq analysis of LNCaP cells expressing designated shRNAs and grown in the presence or absence of DHT. Heatmap shows fold change in gene expression of DEGs in the response to DHT in LNCaP cells expressing shTMEFF2-3 or shScramble shRNA. (**B**) –log10 p-value for enrichment of AR coregulatory genes [19] and LNCaP essential genes [20] among genes significantly upregulated or downregulated by DHT in LNCaP cells expressing shScramble shRNA (Top), and among genes significantly downregulated by shTMEFF2-3 relative to shScramble expressing LNCaP cells (Bottom). –log10 p-values are based on hypergeometric distribution.

A



Comparisons (DHT up, shTMEFF2-3 down)	# Overlapping Gene Sets	-log10(pvalue)
shScramble +DHT vs shScramble -DHT shTMEFF2-3 +DHT vs shScramble +DHT	966	1602
shScramble +DHT vs shScramble -DHT shTMEFF2-3 -DHT vs shScramble -DHT	381	359
shTMEFF2-3 +DHT vs shScramble +DHT shTMEFF2-3 -DHT vs shScramble -DHT	555	1194

Comparisons (DHT down, shTMEFF2-3 up)	# Overlapping Gene Sets	-log10(pvalue)
shScramble +DHT vs shScramble -DHT shTMEFF2-3 +DHT vs shScramble +DHT	1382	1697
shScramble +DHT vs shScramble -DHT shTMEFF2-3 -DHT vs shScramble -DHT	118	0
shTMEFF2-3 +DHT vs shScramble +DHT shTMEFF2-3 -DHT vs shScramble -DHT	465	395

B

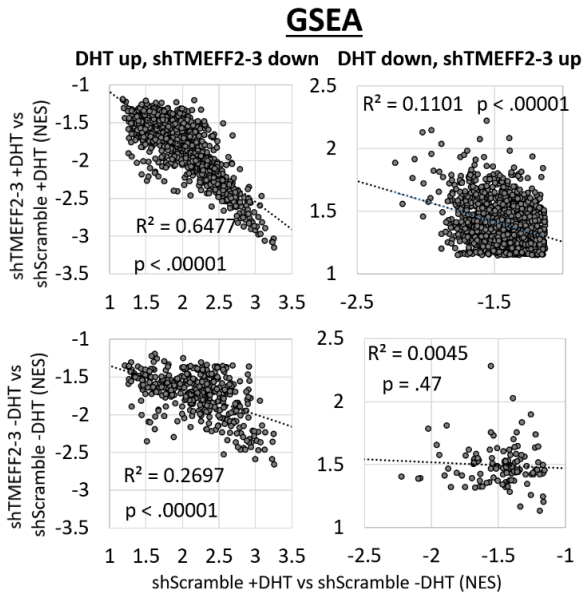


Figure S14. DHT treatment and shTMEFF2-3 oppositely regulate a significant number of gene sets. (**A**) Venn diagrams show the number of gene sets that are significantly downregulated by shTMEFF2-3 or upregulated by DHT in shScramble expressing LNCaP cells (left panel) and significantly upregulated by shTMEFF2-3 or downregulated by DHT in shScramble expressing LNCaP cells (right panel). The number of overlapping gene sets and the significance of the overlap, –log10(p-values) based on hypergeometric distribution, are shown. (shTM: shTMEFF2-3; shScr: shScramble; -: - DHT; +: + DHT). Enriched gene sets were determined by GSEA (q-value < .25) of RNA-seq data. 19,695 total MsigDB gene sets queried. Top 100 DHT upregulated and downregulated gene sets are listed in tables S3 and S4, respectively. (**B**) Correlations of NES values from GSEA of gene sets that exhibit opposite enrichment with shTMEFF2-3 expression and DHT treatment in shScramble expressing LNCaP cells.

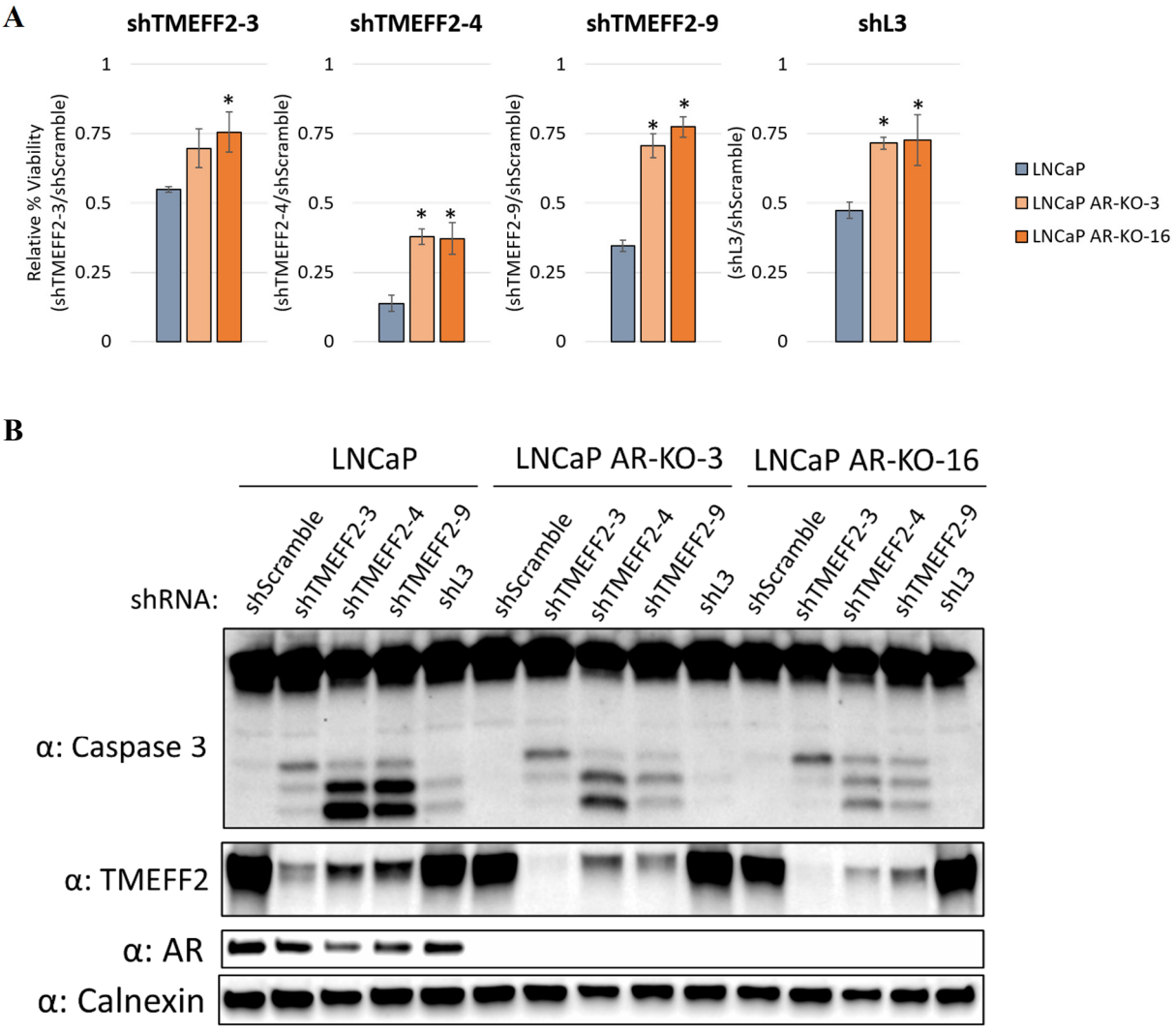


Figure S15. TMEFF2-targeted and shL3 shRNA expression in LNCaP AR-KO cells reduces viability to a lesser degree when compared to LNCaP cells. Relative percent viability (**A**) and western blot showing expression of cleaved Caspase 3, TMEFF2 and AR (**B**) in response to shTMEFF2-3,-4,-9, shL3 and shScramble control shRNA expression in LNCaP and LNCaP AR-knockout cell lines (LNCaP AR-KO-3 and LNCaP AR-KO-16). Percent viability is presented as relative to shScramble control shRNA for each cell line, as determined by trypan blue. N=3, error bars= standard deviation, * $p < .05$ (Relative viability in LNCaP AR-KO compared to LNCaP) determined by t-test.

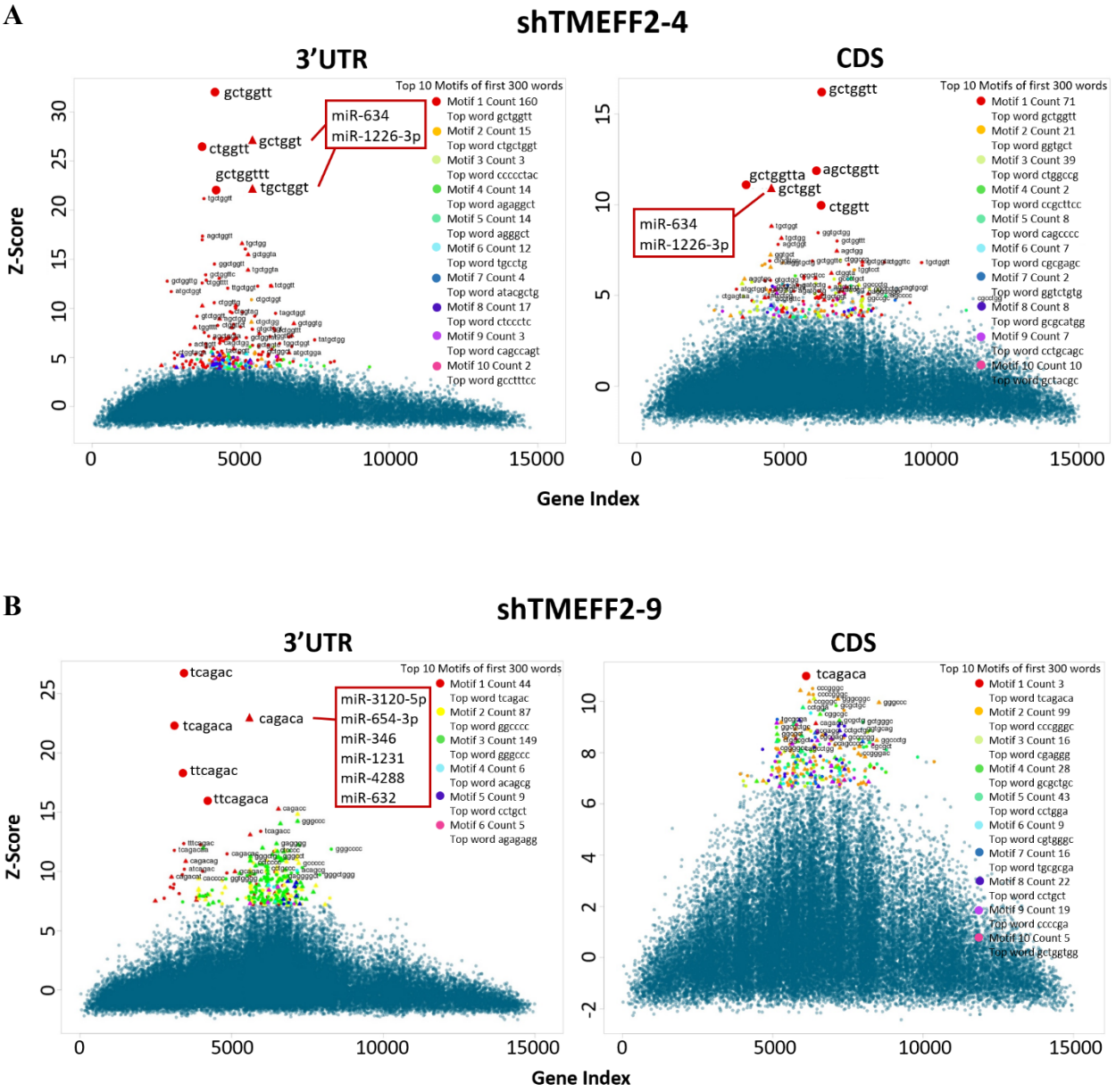


Figure S16. The top 3' UTR motifs associated with shRNA-mediated gene downregulation are complementary to potential shRNA seed sequences. cWords cluster plots showing the Z-scores of the 6mer, 7mer and 8mer nucleotide sequences within the top 10 enriched motifs in the 3' UTR (left panels) or coding sequence (CDS, right panels) of genes downregulated by shTMEFF2-4 (**A**) and shTMEFF2-9 (**B**), shTMEFF2-9 according to RNA-seq analyses. X-axis contains rank ordered genes from the most downregulated to upregulated expression. Each mark represents an individual sequence, and red marks are sequences within the most enriched motifs. Triangles indicate seed sequences of known miRNAs (listed). Circles indicate sequences not currently known to correspond to human miRNA seeds.

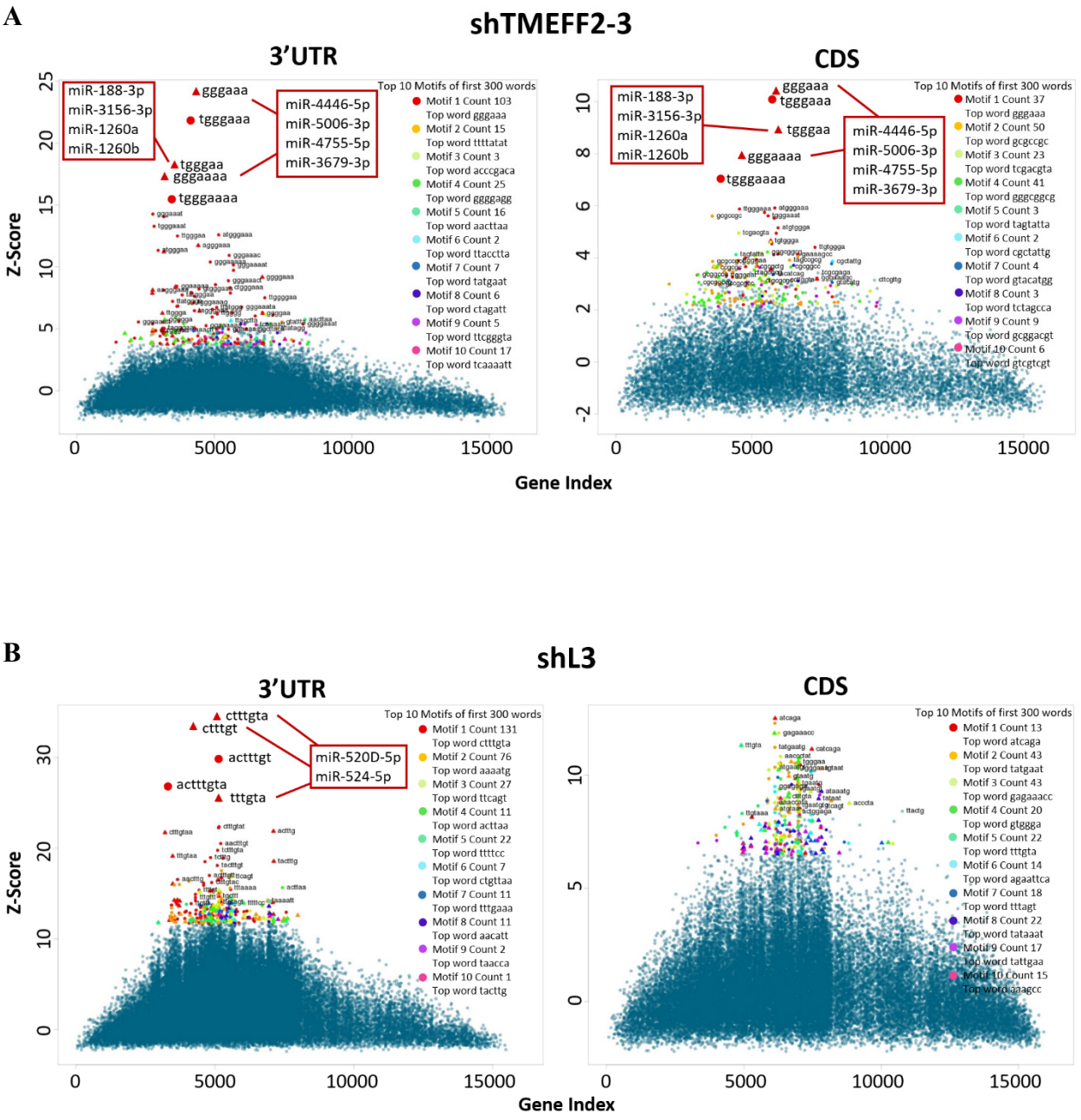


Figure S17. The top 3' UTR motifs associated with shRNA-mediated gene downregulation are complementary to potential shRNA seed sequences. cWords cluster plots showing the Z-scores of the 6mer, 7mer and 8mer nucleotide sequences within the top 10 enriched motifs in the 3' UTR (left panels) or coding sequence (CDS, right panels) of genes downregulated by shTMEFF2-3 (**A**) and shL3 (**B**) according to RNA-seq analyses. X-axis contains rank ordered genes from the most downregulated to upregulated expression. Each mark represents an individual sequence, and red marks are sequences within the most enriched motifs. Triangles indicate seed sequences of known miRNAs (listed). Circles indicate sequences not currently known to correspond to human miRNA seeds.

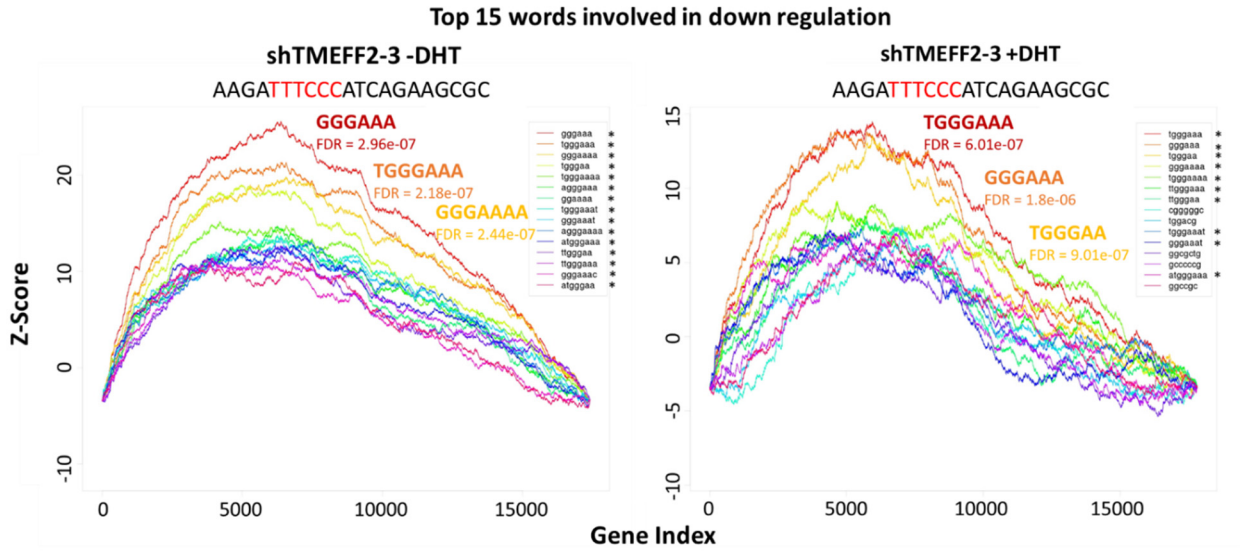


Figure S18. 3' UTR enriched sequences associated with shTMEFF2-3-mediated gene downregulation in cells grown in the presence and absence of DHT are complementary to potential shRNA seed sequences. (**A**) cWords enrichment plots showing the top 15 enriched 6mer, 7mer and 8mer in the 3' UTR of genes downregulated by shTMEFF2-3 in the absence of DHT (left panel) and in the presence of DHT (right panel) according to RNA-seq analyses. Y-axis shows Z-score enrichment values. X-axis contains rank ordered genes from the most downregulated to upregulated expression. The top 3 enriched 3' UTR sequences and FDR values are labeled (red: most enriched, orange: second most enriched, yellow: third most enriched). Unprocessed shTMEFF2-3 guide strand sequence is above each plot, and the potential 6mer seed sequence complementary to the most enriched 6mer 3' UTR sequence associated with gene downregulation is in red. * indicates enriched sequences that are complementary to potential shTMEFF2-3 seed motifs.

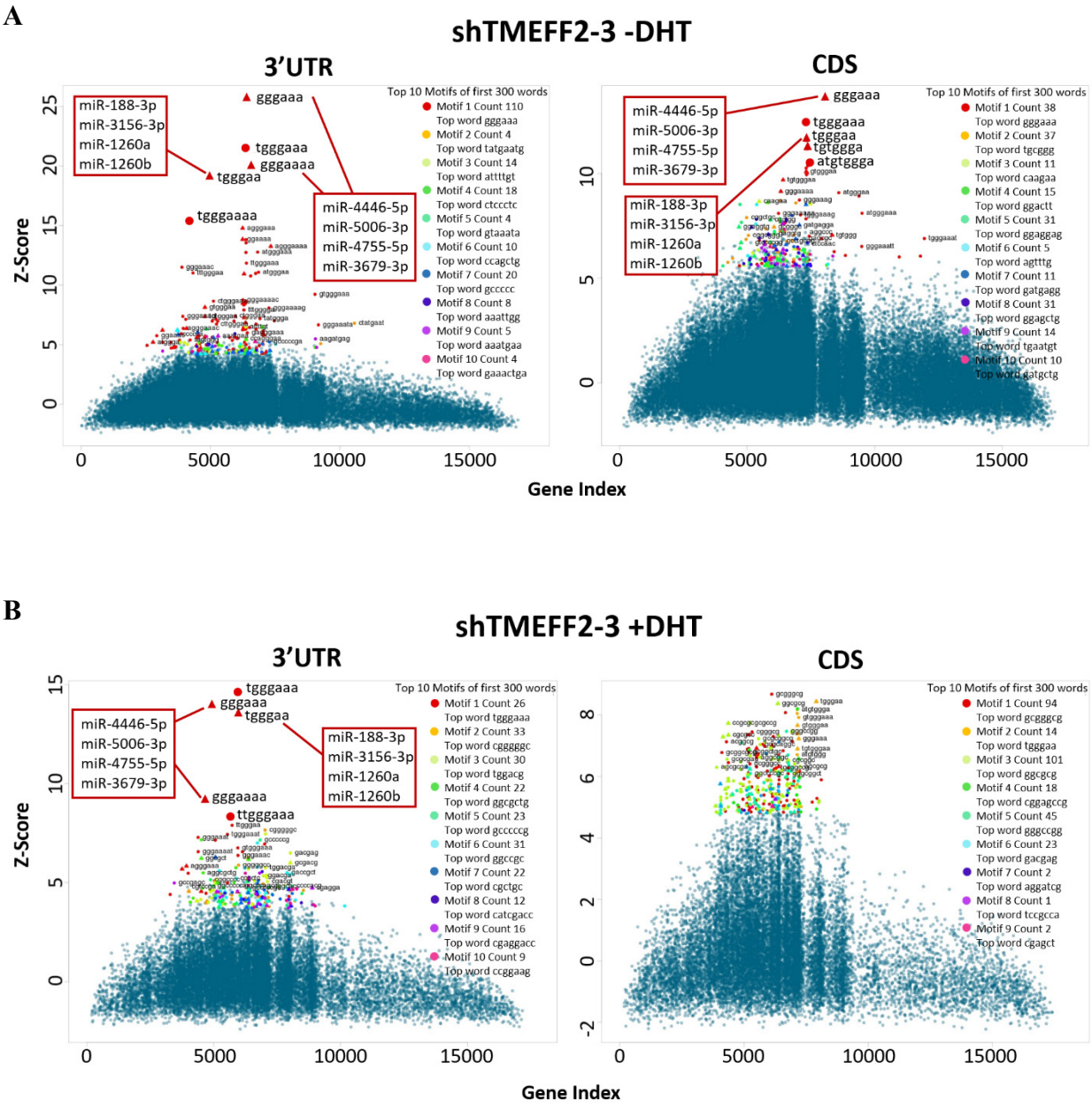


Figure S19. 3' UTR enriched sequences associated with shTMEFF2-3-mediated gene downregulation in cells grown in the presence and absence of DHT are complementary to potential shRNA seed sequences. cWords cluster plots showing the Z-scores of the 6mer, 7mer and 8mer nucleotide sequences within the top 10 enriched motifs in the 3' UTR (left panels) or coding sequence (CDS, right panels) of genes downregulated by shTMEFF2-3 in the absence of DHT (**A**) and in the presence of DHT (**B**) according to RNA-seq analyses. X-axis contains rank ordered genes from the most downregulated to upregulated expression. Each mark represents an individual sequence (red marks are sequences within the most enriched motifs). Triangles indicate seed sequences of known miRNAs (listed). Circles indicate sequences not currently known to correspond to human miRNA seeds.

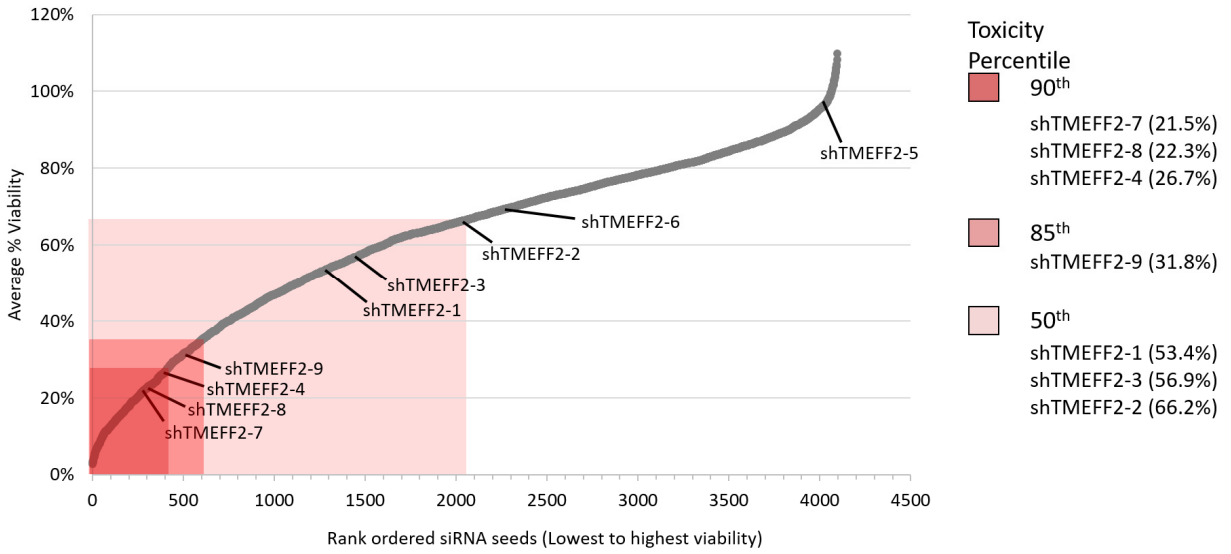


Figure S20. *In silico* analysis of siRNA seed viability screen reveals enrichment of potential TMEFF2 shRNA seeds within the most toxic seeds. Dot plot showing the average viability of HeyA8 and H460 cells transfected with siRNAs containing all possible (4096) 6mer seed sequences (data from Gao et al [22]). Seeds are rank ordered from effecting the lowest to highest viability and red rectangles mark percentile rankings of seed toxicity (90^{th}, 85^{th}, and 50^{th} percentile). Average percent viability of TMEFF2-targeted shRNA seeds, assuming consistent Dicer cuts during shRNA processing, are indicated only for those shRNA seeds within the 50^{th} percentile of toxicity.

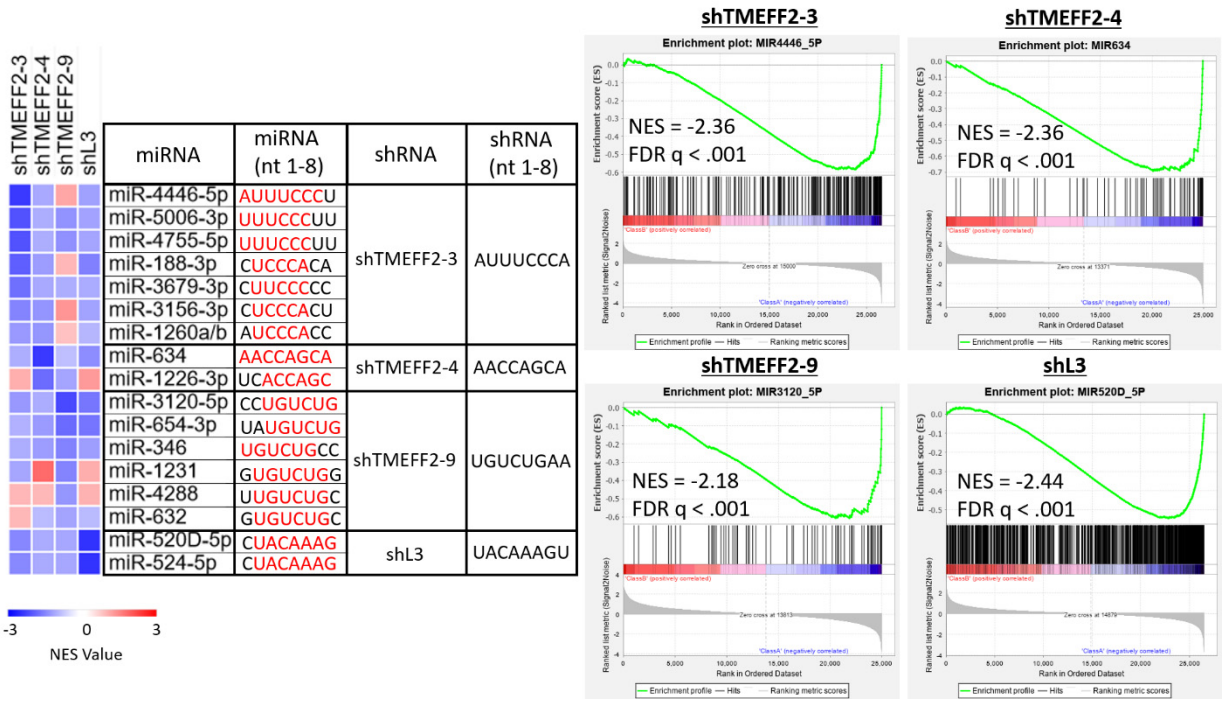


Figure S21. Gene sets of potential miRNA targets are downregulated by shRNAs with similar seed sequences. GSEAs of miRDB predicted target gene sets of miRNAs with seed motifs similar to shTMEFF2-3, -4, -9 or shL3 (as predicted by cWords analyses). Heatmap (left panel) shows the NES of each gene set. Positive NES indicates gene sets are upregulated, and negative NES indicates gene sets are downregulated by the designated shRNA. The first eight nucleotides of the guide strand of miRNAs and shRNAs are listed, and identical miRNA and shRNA sequences are in red. GSEA enrichment plots (right panel) showing the enrichment of the most significantly enriched miRDB gene set in rank ordered gene expression lists (upregulated to downregulated) for each shRNA. NES and FDR q-values are labeled on each plot.

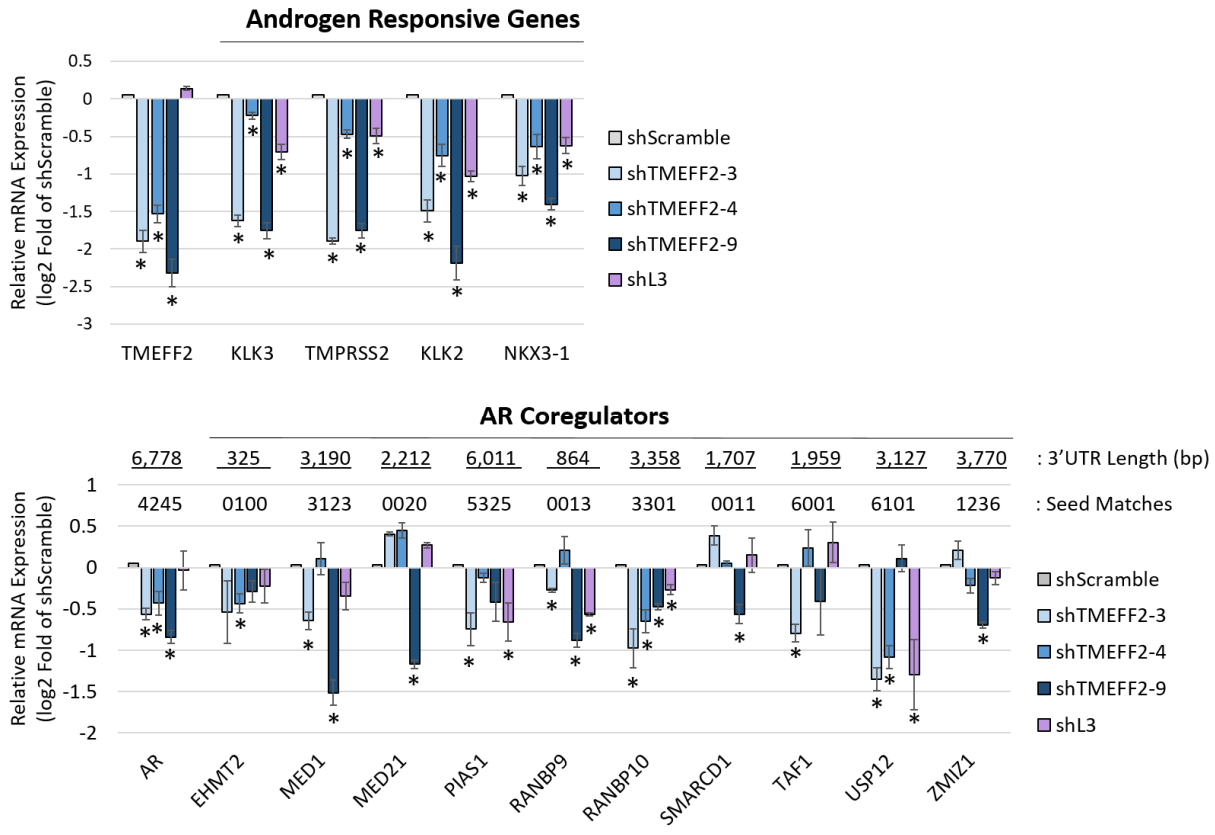
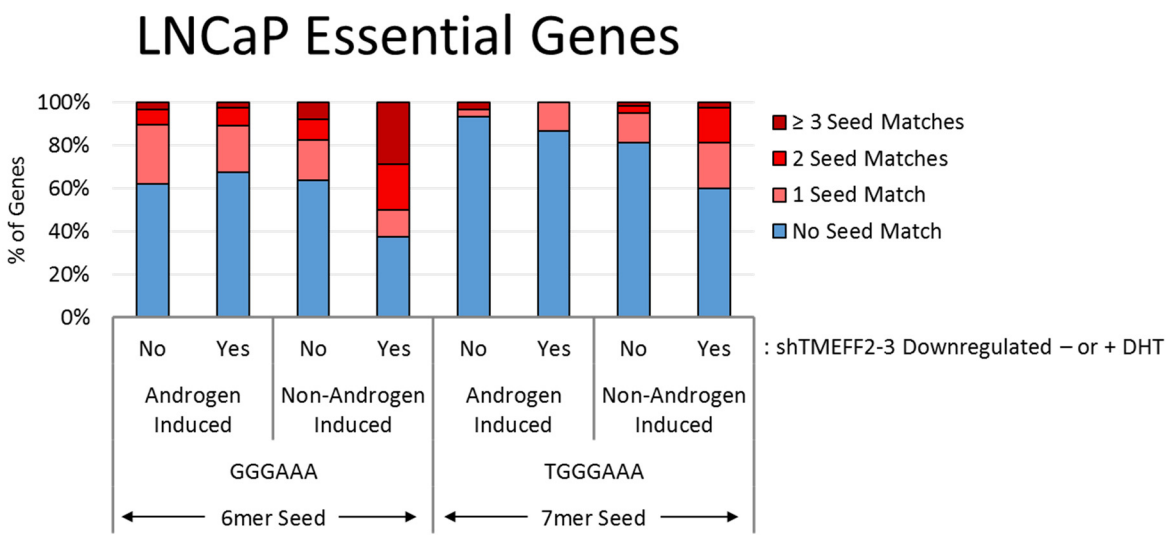


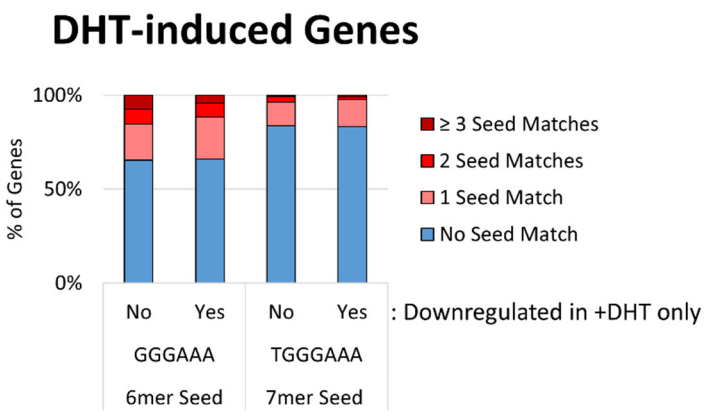
Figure S22. RT qPCR validation of androgen responsive and AR coregulatory gene downregulation by shRNAs. mRNA expression validation of androgen responsive (top panel) and AR coregulatory (bottom panel) gene expression. Genes selected were downregulated in LNCaP cells expressing the designated shRNAs based on RNA-seq analyses. Shown is the relative mRNA expression determined by RT qPCR (as log2 fold of shScramble). 3' UTR length and number of 3' UTR sequences complementary to 6mer seeds for each shRNA are labeled. RPL8, RPL38, PSMA1 and PPP2CA were housekeeping genes used for normalization. N=3, error bars ±SD, * $p<.05$ determined by t-test.



		Androgen Induced		Non-Androgen Induced	
		shTMEFF2-3 Down		shTMEFF2-3 Down	
		No	Yes	No	Yes
6mer Seed Match	No	18	25	542	30
	Yes	11	12	311	50
		χ^2 = 0.216		χ^2 = 20.91	
		p = .642		p = 4.81E-06	

		Androgen Induced		Non-Androgen Induced	
		shTMEFF2-3 Down		shTMEFF2-3 Down	
		No	Yes	No	Yes
7mer Seed Match	No	27	32	693	48
	Yes	2	5	160	32
		χ^2 = 0.751		χ^2 = 20.19	
		p = .386		p = 7.01E-06	

Figure S23. Essential gene downregulation is associated with 3' UTR complementary to sh-TMEFF2-3 seed sequence, and/or androgen signaling inhibition. LNCaP essential gene set [20] stratified by whether genes are induced by DHT (Androgen induced or Non-Androgen induced, based on data in shScramble cells), downregulation by shTMEFF2-3 (yes or no), and by the presence in their 3'-UTR of single, 2, or more, 6mer or 7mer sequences identified by cWords analyses. Contingency tables are located below each stacked bar graph. P-values were calculated by chi square test of independence, and are labeled on the bottom of each contingency table.



		shTMEFF2-3 Down +DHT only	
		No	Yes
6mer Seed Match	No	335	223
	Yes	178	115
		χ^2 = .04	
		p = .840	

		shTMEFF2-3 Down +DHT only	
		No	Yes
7mer Seed Match	No	430	281
	Yes	83	57
		χ^2 = .069	
		p = .792	

Figure S24. Downregulation of AR signaling regulated genes by shTMEFF2-3 is not associated with 3' UTR complementary to sh-TMEFF2-3 seed sequence. Genes significantly upregulated by DHT treatment in LNCaP cells expressing shScramble shRNA in RNA seq analysis were stratefied by downregulation by shTMEFF2-3 in the presence of DHT only (338 out of 455 downregulated by shTMEFF2-3) (yes or no), and by the presence in their 3'-UTR of single, 2, or more, 6mer or 7mer sequences identified by cWords analyses. Contingency tables are located below each stacked bar graph. P-values were calculated by chi square test of independence, and are labeled on the bottom of each contingency table.

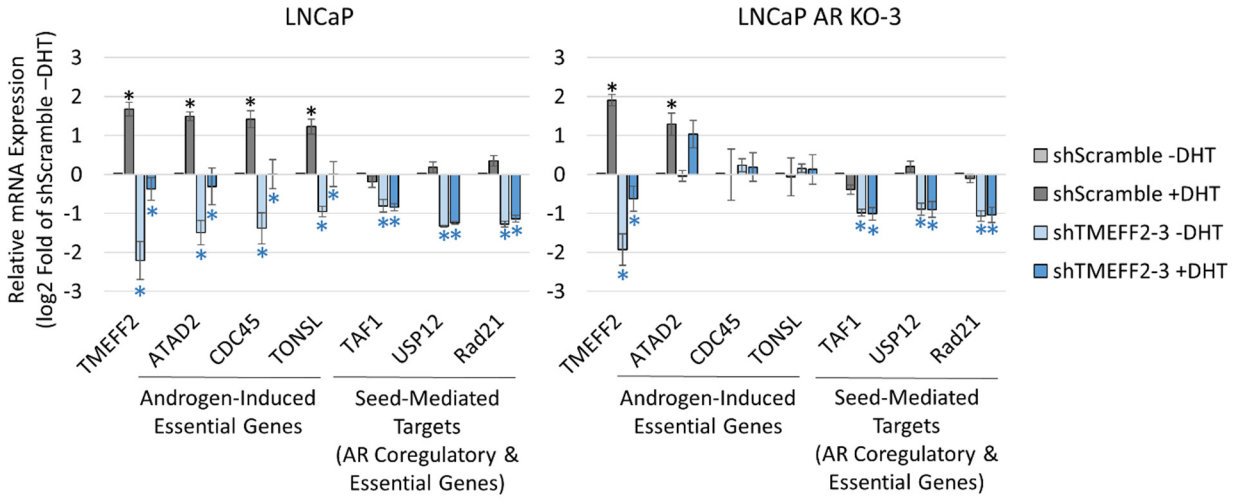


Figure S25. TMEFF2-targeted shRNA expression in LNCaP AR-KO cells reduces the expression direct seed-mediated essential and AR coregulatory gene targets, with no effect on the expression of AR-signaling responsive essential genes (lacking seed matches). Relative mRNA expression of TMEFF2, androgen-induced essential genes, and non-androgen regulated AR coregulatory and essential genes in LNCaP and LNCaP AR-KO-3 cells expressing shTMEFF2-3 or shScramble and grown in the presence and absence of 10 nM DHT. Genes were identified as being androgen induced and/or downregulated by shTMEFF2-3 in RNA seq analysis of LNCaP cells expressing shScramble or shTMEFF2-3 and grown in the presence and absence of DHT. mRNA expression is relative to shScramble expressing cells in the absence of DHT for each cell line. N=3, error bars = standard deviation. * $p < .05$ (black *: shScramble +DHT vs shScramble –DHT; blue *: shTMEFF2-3 vs shScramble in + or – DHT) determined by t-test.

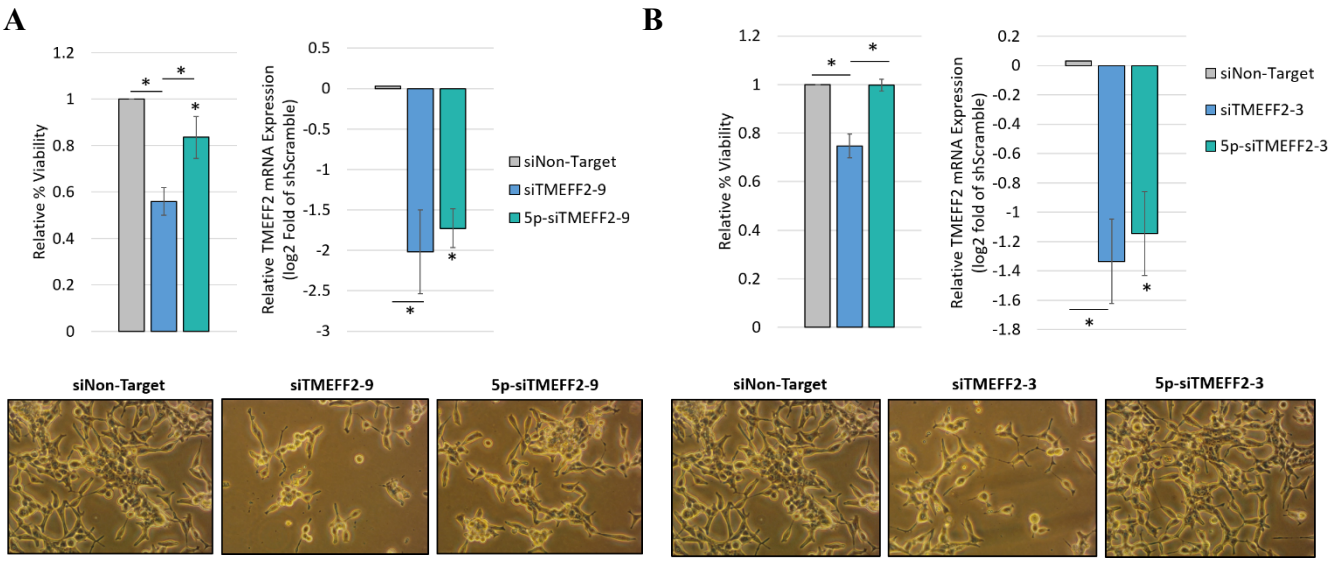


Figure S26. Reductions in PCa cancer cell viability are seed mediated. Relative percent viability and TMEFF2 mRNA expression in LNCaP cells transfected with siTMEFF2-9 (**B**) or siTMEFF2-3 (**C**) and control siRNAs (siNon-Target; Negative seed control: 5p-siTMEFF2). 5p designates an ON-Target-Plus modification (Dharmacon) that blocks seed mediated gene downregulation. Cell viability was determined by trypan blue. Viability measurements, cell pictures and RNA extractions were done 72 hours after siRNA transfections. mRNA expression was determined by RT qPCR using RPL8, RPL38, PSMA1 and PPP2CA housekeeping genes for normalization. N=4, error bars ±SD, * $p<.05$ compared to siNon-target. Bars with * designate significant differences relative to siTMEFF2-9 or siTMEFF2-3. Significance was determined by t-test.

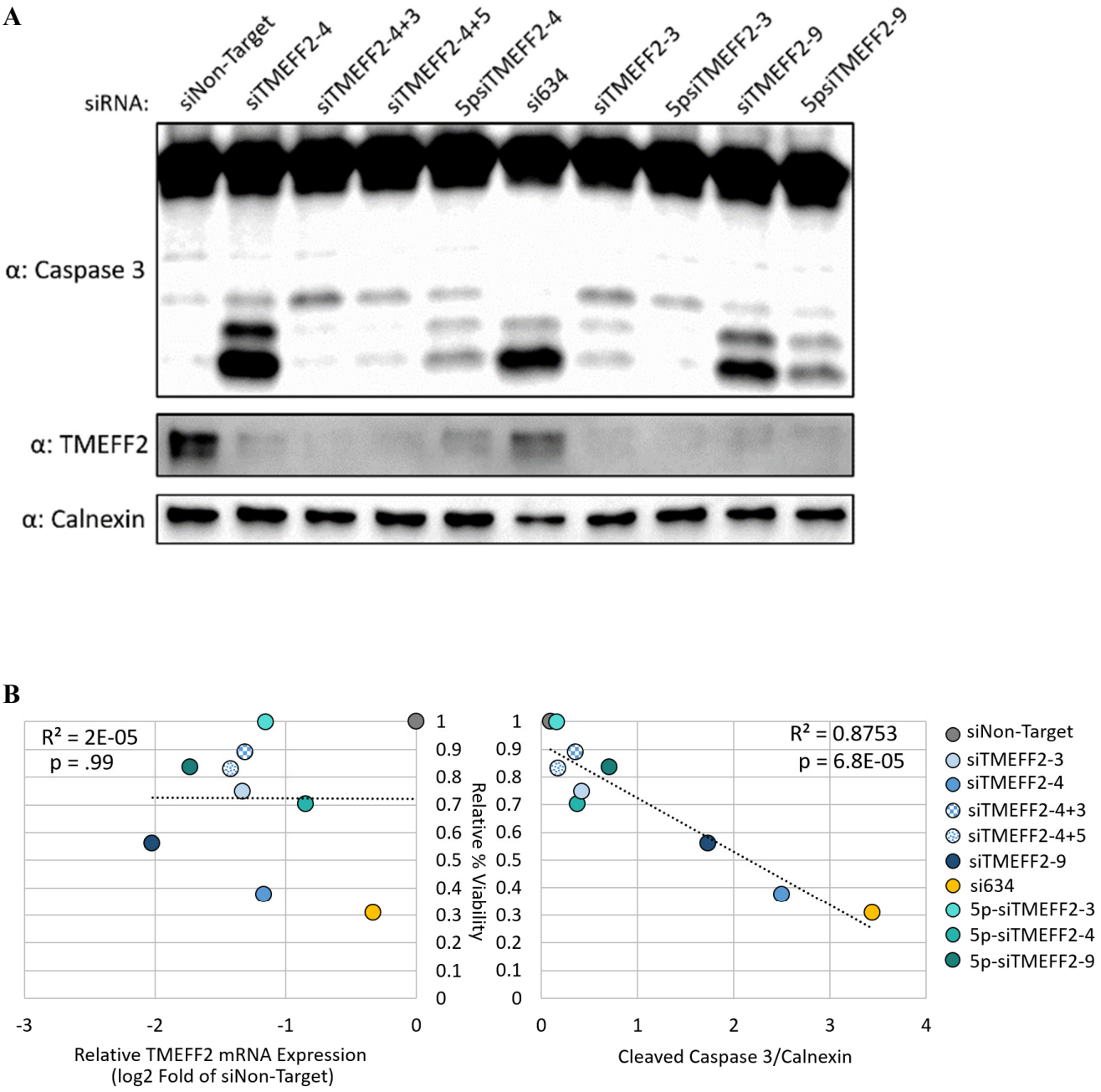
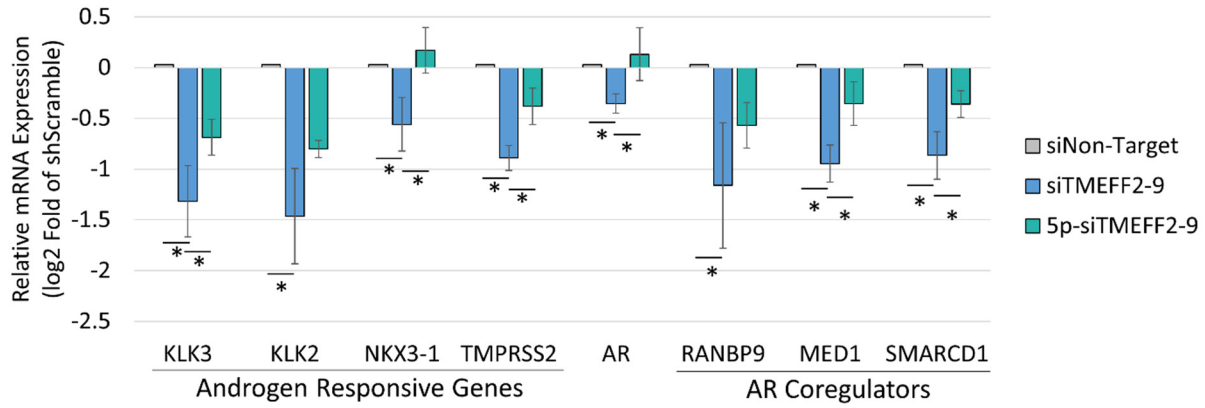


Figure S27. Increased Caspase 3 cleavage is mediated by siRNA seed sequence independently of TMEFF2 expression levels (**A**) Western blot analysis showing Caspase 3 cleavage and TMEFF2 protein expression in lysates from LNCaP cells transfected with the designated siRNAs. Lysates were obtained 72 hours after siRNA transfections. Calnexin was used as a loading control. (**B**) Correlations of relative percent viability with relative TMEFF2 mRNA expression (left) and normalized cleaved Caspase 3 (right) in LNCaP cells transfected with designated siRNAs. Band intensity was quantified using Biorad Image Lab. See Figure 5A for viability and TMEFF2 mRNA levels.

A



B

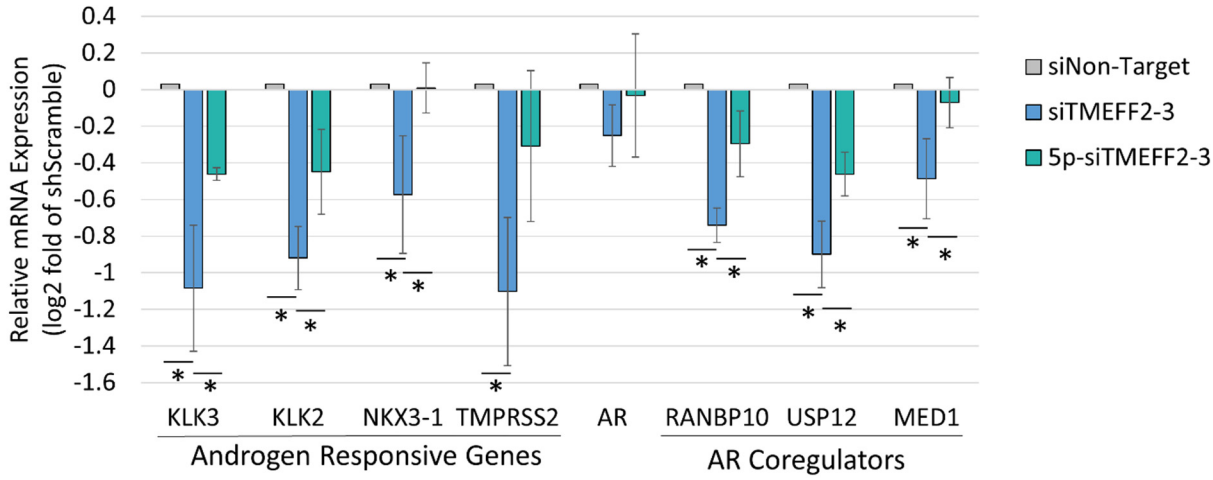


Figure S28. AR coregulatory gene downregulation are seed mediated. Androgen responsive, AR coregulator and AR mRNA expression in LNCaP cells transfected with siTMEFF2-9 (**A**) or siTMEFF2-3 (**B**) and control siRNAs (siNon-Target; Negative seed controls: 5p-siTMEFF2-3). 5p designates an ON-Target-Plus modification (Dharmacon) that blocks seed mediated gene downregulation. mRNA expression was determined by RT qPCR using RPL8, RPL38, PSMA1 and PPP2CA housekeeping genes for normalization. N=4, error bars ±SD, * $p<.05$ determined by t-test.

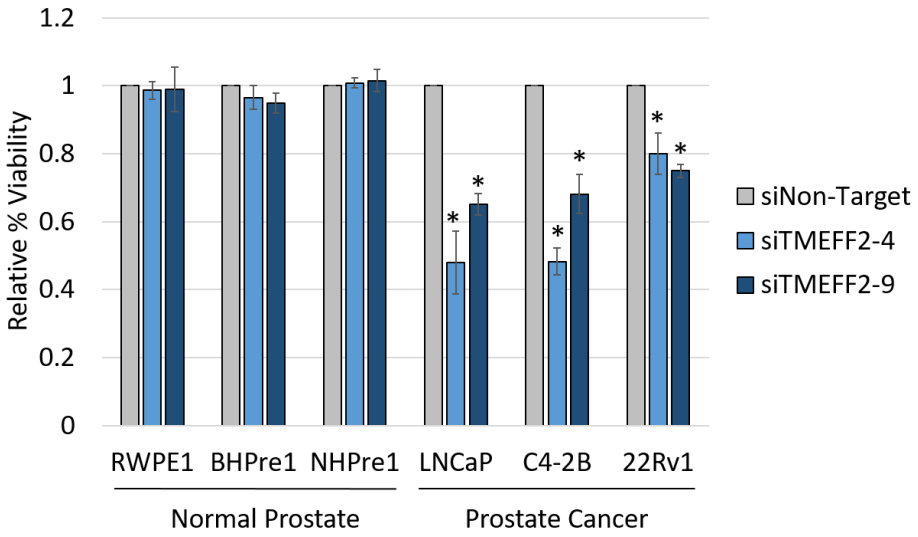


Figure S29. **Toxic siRNAs do not reduce the viability of normal prostate epithelial cell lines.** Relative percent viability of normal prostate (RWPE1, BHPre1, NHPre1) and PCa cell lines (LNCaP, C4-2B and 22Rv1) transfected with Cy5 labeled siNon-Target, siTMEFF2-4 or siTMEFF2-9 siRNAs. Viability was determined by trypan blue 72 hours after siRNA transfection. N=3, error bars ±SD, * $p<.05$ determined by t-test.

Table S1. Transcripts identified by deep RNA-seq of TMEFF2 locus and the number of shRNAs targeting each transcript. Table shows four TMEFF2 isoforms and ten lncRNAs detected as being expressed from the TMEFF2 locus in LNCaP cells using deep RNA-seq analysis (317 million good reads). A list of detected transcripts is provided along with information regarding location, transcript/gene ID/symbol, length, number of exons, relative transcript abundance (coverage/ Fragments Per Kilobase of transcript per Million mapped reads (FPKM)), and the number of shRNA target sequences out of nine total TMEFF2-targeted shRNAs.

Chr	strand	Hg38 Start	End	Transcript ID	# exons	Length	Gene ID	Gene Symbol	Coverage	FPKM	# shRNA Target Sequences
2	-	191950017	192195709	ENST00000272771.9	10	2604	ENSG00000144339.11	TMEFF2	605.5575	63319.688	9
2	-	191949043	192194916	ENST00000392314.5	10	2842	ENSG00000144339.11	TMEFF2	263.03003	27503.549	8
2	-	192176743	192194524	ENST00000409056.3	4	3364	ENSG00000144339.11	TMEFF2	65.105675	6807.7285	2
2	+	192032548	192044525	lnc-OBFC2A-5:20	3	1152	lnc-OBFC2A-5	.	23.760929	2484.5447	0
2	+	192035256	192037103	ENST00000625221.1	3	805	ENSG00000233766.7	AC098617.1	21.792868	2278.7559	0
2	+	192021912	192029036	lnc-OBFC2A-5:4	3	277	lnc-OBFC2A-5	.	21.315186	2228.8071	0
2	+	192032368	192035380	ENST00000598327.3	3	638	ENSG00000233766.7	AC098617.1	18.98439	1985.0894	0
2	-	191992664	191999205	ENST00000487771.1	3	278	ENSG00000144339.11	TMEFF2	12.179338	1273.5238	1
2	+	192036170	192038059	lnc-OBFC2A-5:23	4	624	lnc-OBFC2A-5	.	11.182352	1169.2748	0
2	+	191922546	192036829	ENST00000428980.6	5	1207	ENSG00000233766.7	AC098617.1	10.120426	1058.2352	0
2	+	192035210	192037121	ENST00000602099.3	4	708	ENSG00000233766.7	AC098617.1	0.411862	43.066055	0
2	+	192035210	192037121	lnc-OBFC2A-5:21	4	708	lnc-OBFC2A-5	.	0.346128	36.192627	0
2	+	192035256	192037103	lnc-OBFC2A-5:22	3	805	lnc-OBFC2A-5	.	0.287261	30.037245	0
2	+	192030605	192036747	ENST00000599681.5	5	767	ENSG00000233766.7	AC098617.1	0.002465	0.257751	0

Table S2. Common enriched gene sets resulting from shTMEFF2-3, shTMEFF2-4, shTMEFF2-9 and shL3 expression in LNCaP cells. Normalized enrichment scores (NES) and q-values for significantly enriched gene sets (GSEA; q-value <.25, for each shRNA) common to LNCaP cells expressing the designated shRNAs compared to cells expressing the shScramble control. q-value=0 : <.001.

Table S3. shTMEFF2-3 regulation of the top 100 DHT upregulated gene sets. Table showing the top 100 gene sets (based on GSEA NES value) upregulated by DHT treatment in shScramble LNCaP cells. NES and q-values are shown for gene set regulation by DHT (shScramble +DHT vs shScramble –DHT) and by shTMEFF2-3 in the presence of DHT (shTMEFF2-3 +DHT vs shScramble + DHT). Positive NES values indicate gene set upregulation. Negative NES values indicate gene set downregulation. q-value < .25 indicates significantly enriched gene set. q-value=0 : <.001.

Table S4. shTMEFF2-3 regulation of the top 100 DHT downregulated gene sets. Table showing the top 100 gene sets (based on GSEA NES value) downregulated by DHT treatment in shScramble LNCaP cells. NES and q-values are shown for gene set regulation by DHT (shScramble +DHT vs shScramble –DHT) and by shTMEFF2-3 in the presence of DHT (shTMEFF2-3 +DHT vs shScramble +DHT). Positive NES values indicate gene set upregulation. Negative NES values indicate gene set downregulation. q-value < .25 indicates significantly enriched gene set.

Table S5. AR Coregulatory genes that contain 3'UTR seed matches and are downregulated by shTMEFF2-3, shTMEFF2-4, shTMEFF2-9, shL3. Table shows AR coregulatory genes that contain 1 or more 3' UTR seed matches and were downregulated (log2 fold change ≤ -.5, adjusted p-value < .05) by each shRNA in RNA seq analysis.

shTMEFF2-3		shTMEFF2-4		shTMEFF2-9		shL3	
Gene Name	**# 3' UTR Seed Matches**	**Gene Name**	**# 3' UTR Seed Matches**	**Gene Name**	**# 3' UTR Seed Matches**	**Gene Name**	**# 3' UTR Seed Matches**
CCND3	1	BRCA1	1	APPBP2	1	GAK	1
FKBP5	1	CALM1	1	BAG1	1	KAT2B	1
HELZ2	1	CALR	1	CALM1	1	CALM1	2
HIPK3	1	CDC25A	1	HIPK3	1	CASP7	2
PER1	1	CTNNB1	1	KAT2B	1	CDK2AP1	2
PIK3CB	1	EHMT2	1	PMEPA1	1	ETV1	2
PPP2R1A	1	FKBP4	1	RANBP9	1	HTATIP2	2
YWHAH	1	IDE	1	RPS6KA3	1	NLK	2
APPBP2	2	KAT2B	1	SMARCD1	1	PXN	2
CDK7	2	PSMC3IP	1	SRCAP	1	STAT5B	2
ADAM10	3	SMAD1	1	ADAM10	2	HDAC4	3
MED1	3	CCND1	2	GSK3B	2	RANBP9	3
RANBP10	3	RPS6KA1	2	HIP1	2	RNASEL	3
TPD52	3	CTDSP2	3	IL6ST	2	APPBP2	4
CTDSP2	4	RANBP10	3	MED1	2	PIAS1	5
PIAS1	5	RPS6KA3	3	MED21	2	SMAD4	6
TAF1	6	MAPK1	4	MKRN1	2	ADAM10	7
USP12	6	PIK3R1	4	UBE2L3	2	MAPK1	7
				ZMIZ1	3	CDK6	9
				NCOA2	4	EGFR	10
				MAPK1	5		