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TNK2 Tyrosine Kinase as a Novel Therapeutic Target in Triple-Negative Breast Cancer

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14. ABSTRACT Triple-negative breast cancers (TNBCs) represent only 10%-15% of all breast cancers; however, they are highly aggressive and have a higher rate of metastasis and a poorer prognosis than other breast cancer subtypes. The primary reason contributing to the poor clinical outcomes associated with TNBCs is the lack of clearly defined therapeutic targets. We observed a novel non-receptor tyrosine kinase, TNK2 (also known as ACK1), to be hyperphosphorylated and activated in highly aggressive TNBC cells. Suppression of TNK2 by specific siRNAs significantly reduced the proliferation, colony formation, and invasive ability of TNBC cells. Our proposed study will allow us to validate TNK2 as novel therapeutic target for TNBC patients. Novel breast cancer therapies directed against TNK2 can be developed based on our discoveries, which can decrease the recurrence and metastatic disease of TNBCs and eventually reduce breast cancer death. The objective of this proposal is to evaluate the therapeutic potential of TNK2 in the treatment of TNBCs. In parallel, pharmacological inhibitor, DZ1-067 will be evaluated as a therapeutic target in vitro and in preclinical animal models. To elucidate the signaling network of TNK2 in oncogenesis of TNBCs, we will perform a LC-MS/MS-based comprehensive phosphoproteomic study to identify signaling cascades modulated by TNK2. This study will define TNK2 as a novel therapeutic target for TNBCs.					
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BC142318P1. TNK2 Tyrosine Kinase as a Novel Therapeutic Target in Triple Negative Breast Cancer (W81XWH-15-1-0312)

INTRODUCTION

Heterogeneity of Breast cancer is reflected in realization of various subtypes that are based on expression of of estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor. About ~15% of invasive breast cancers do not express these receptors and are termed as Triple negative breast cancer (TNBC), which are often associated with a poor prognosis [1-4]. Unlike HER2/*neu* amplified or ER positive cancers that has been successfully treated with endocrine therapy [5] or HER2-targeted therapy [6], TNBCs are very difficult to treat because of the lack of ER, PR or HER2 expression. Recently, expression of multiple receptor tyrosine kinases including EGFR, MET and c-Kit have been shown to be upregulated in subsets of TNBCs [7, 8], however, monoclonal anti-EGFR antibody cetuximab, alone or in combination with cytotoxic chemotherapies, showed minimal improvement in progression-free and overall survival of TNBC patients [9, 10].

Interestingly, our quantitative mass spectrometry-based phosphoproteomics profiling done in 26 TNBC cell lines led to identification non-receptor tyrosine kinase, TNK2, also known as ACK1, to be the protein that was hyperphosphorylated in a majority of TNBCs [11]. TNK2/ACK1 has earlier shown to be activated (by RTKs) and amplified or mutated in multiple cancers including prostate, lung, breast, esophageal, ovarian, melanoma and pancreatic cancer [12, 13]. Overexpression of TNK2 results in increased invasive phenotype, leading to increased mortality in mouse model of metastasis [12, 14]. Further, expression of activated TNK2/ACK1 was positively correlated with the severity of disease progression, and inversely correlated with the survival of breast cancer patients [15]. Being a cytoplasmic kinase, TNK2/ACK1 integrates extracellular signals from multiple receptor tyrosine kinases including EGFR, HER2 and PDGFR to intracellular effectors [14-17]. Significantly, TNK2/ACK1 has shown to directly phosphorylate AKT at Tyr-176, consequently promoting Thr-308 and Ser-473 phosphorylation, leading to AKT activation [15]. In addition, TNK2/ACK1 binds and directly phosphorylates androgen receptor or AR at Tyr-267 and Tyr-363 in prostate cancer cells facilitating androgen-independent transcriptional activation of AR [17]. In breast cancer, TNK2/ACK1 exhibited phosphorylation of the ER co-activator, KDM3A an H3K9 demethylase at Tyr-1114, leading to upregulation of a mammary tumor oncogene, HOXA1, conferring tamoxifen resistance [18].

Although, there were indications that activation of TNK2 may have role in breast cancer [15, 19], the functional significance of TNK2 expression in TNBCs was not fully explored. This proposal addressed these shortcoming. Dr. Juan Carlos Roa, our collaborating pathologist at the School of Medicine in Pontificia Universidad Católica de Chile in Santiago, Chile has assembled a set of breast cancer TMAs comprising >300 breast cancer tumor cores that includes almost 100 TNBCs, with clinical outcomes. We have used those TMAs for ACK1 as well as phospho-ACK1 staining.

Recently, we have reported a new class of robust and soluble TNK2/ACK1-specific small molecule inhibitor, (R)-9bMS, also known as DZ1-067 [20, 21]. Using (R)-9bMS we were able to show that TNK2-AKT signaling may play an important role in TNBC growth and proliferation. Further, it opened a new and promising therapeutic strategy, treatment of TNBCs with (R)-9bMS.

Note: The TMAs used in these studies were generated using samples that were obtained from Chile. Which created significant issues in getting IRB approval. Since, we could not obtain IRB approval for more than one year, the experiments described below in Major Task 1 (Subtask 1 and 2), which includes staining of TMA (Subtask 1) and further analysis of TMA data (Subtask 2) were significantly delayed. We asked for the extension of another 1 year to complete the experiments, especially Subtask 2. However, because of recent move by PI (Dr Nupam Mahajan) from Moffitt cancer Center to Washington University at St. Louis (in August 2018), the extension of this grant was denied.

In that process, PI has ended up returning large portion of funds back to DoD; \$373,000 of the total \$589,750 (approximately 63%) were returned back. In spite of returning more than 60% of the grant funds, PI has accomplished all the proposed studies, except parts of Subtask 2.

BODY

RESULTS

Specific Aim 1: Does TNK2 protein levels and activation correlate with clinical and pathological features of TNBC?

Major Task 1: To determine TNK2 activation using IHC in retrospective primary breast cancer tissue specimens with clinical outcomes data.

Subtask 1: To perform IHC study on breast cancer TMAs with phospho TNK2 specific antibody

Completed: 100%

We performed pY284-TNK2 and TNK2 antibody characterization in IHC. In brief, previously obtained breast cancer high-density TMA containing samples of normal breast tissue, atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), lymph node macro metastasis (LNMM) was used. Four mm sections were cut with Leica microtome (Leica Microsystems Inc, Bannockburn, IL) and transferred to adhesive-coated slides. The tissue array slides (4 slides including 2 test duplicate slides, and positive and negative controls) were stained for pTyr284-TNK2 using respective rabbit polyclonal antibodies.

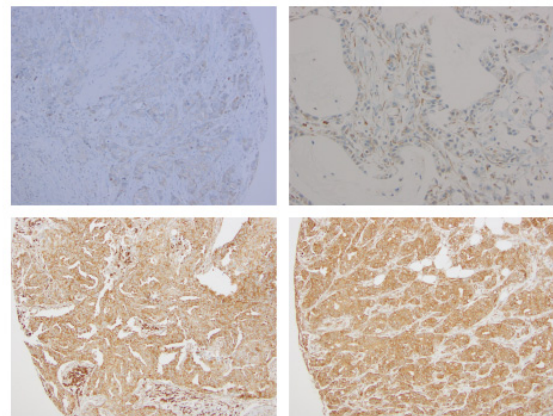


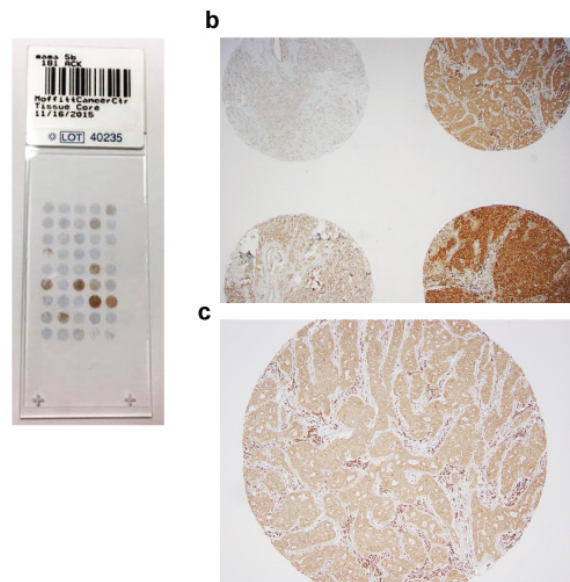
Figure 1. Detection of TNK2 expression in TNBC by IHC. TNBC TMA slide stained with TNK2 antibodies. A representative staining pattern in different stages is shown.

The slides were dewaxed by heating at 55 Celsius for 30 min and by three washes, 5 min each, with xylene. Tissues were rehydrated by series of 5 min washes in 100%, 95%, and 80% ethanol and distilled water. Antigen retrieval was performed by heating the samples at 95o Celsius for 30 min in 10 mmol/L sodium citrate (pH 6.0). After blocking with universal blocking serum (DAKO Diagnostic, Mississauga, Ontario, Canada) for 30 min, the samples were then incubated with rabbit polyclonal pTyr284-TNK2 antibody (1:300 dilution; Milipore). The sections were incubated with biotin-labeled secondary and streptavidin-peroxidase for 30 min each (DAKO Diagnostic). The samples were developed with 3,39-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) and counterstained with hematoxylin.

Following standard procedures the slides were dehydrated and sealed with cover slips. Negative controls were included by omitting pTyr284-TNK2 antibody during primary antibody incubation. The phospho-TNK2 antibodies were extensively validated for immunohistochemistry studies.

We found a significant number of TNBCs that were strongly, moderately or weakly positive for TNK2 and pTNK2 staining. A representative TMA slide stained with pTNK2 antibodies is shown in **Figure 1** and **2**.

Figure 2. Detection of phospho-TNK2 expression in TNBC TMA. (a) A representative TNBC TMA slide stained with phospho-TNK2 antibodies is shown. (b and c) The enlargement of TNBC TMA slide stained with phospho-TNK2 antibodies.



Subtask 2: To score the IHC staining results and perform statistical analysis based on pathological features and clinical outcomes

Completed: 50%

To address clinical relevance of TNK2 expression, 91 TNBC samples were stained with TNK2/ACK1 antibodies followed by reading of the slides by pathologist. The data is shown in form of pie chart (Figure 3).

About 6% of the patients stained strongly for TNK/ACK1, while 14% of the patients were moderately positive, while 22% of patients were weakly positive for ACK1 expression. Little over half patients were negative for ACK1 expression.

In addition, we have also performed staining of these TMAs with pY284-ACK1 (phospho-ACK1) antibodies. Unfortunately, the pathologist have not yet been able to read these slides (the reasons are discussed above).

However, we hope to pursue these studies and complete the reading of TMA, followed by detailed statistical analysis to assess correlation between activation of TNK2 and disease progression. These data will be published in course of time.

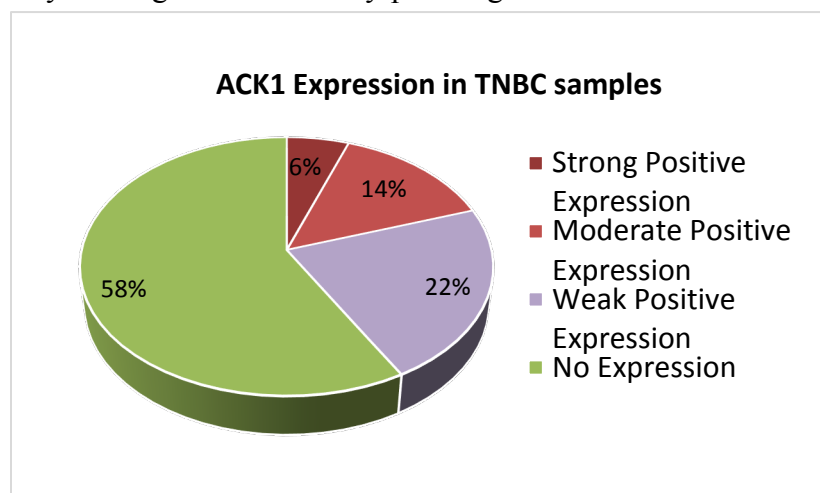


Figure 3. TNK2/ACK1 expression in human TNBCs. A relative expression of ACK1 in TNBCs is shown.

Subtask 3: To suppress TNK2 activity by TNK2 specific inhibitor, DZ1-067 and perform functional assays

Completed: 100%

The aim of this Subtask was to assess whether TNK2 specific inhibitor, DZ1-067, also known as (*R*)-9bMS suppresses TNK2 activity, resulting in significant loss of its downstream substrate AKT. We not only assessed loss of AKT Tyr176-phosphorylation upon (*R*)-9bMS treatment of TNBCs, but also examined its effect on TNBC proliferation.

Ability of TNK2 to directly phosphorylate AKT at Tyr176 site leading to its activation indicates that TNK2-AKT signalling nexus may be involved in driving TNBC tumorigenicity. We have reported a new class of robust and

soluble TNK2-specific small molecule inhibitor, (*R*)-9bMS [20, 21]. To address role of TNK2 in AKT Tyr176-phosphorylation in TNBCs, cells were treated with (*R*)-9bMS. A significant decrease in TNK2 activation, as seen by loss of TNK2 Tyr-phosphorylation is observed upon (*R*)-9bMS treatment (**Figure 4A**). Inhibition of TNK2 activation is also reflected in significant decrease in AKT Tyr176-phosphorylation (**Figure 4B**). Further, several TNBC cell lines were found to be sensitive to the (*R*)-9bMS treatment with IC₅₀ of 0.45 μ M (MDA-MB-231), 0.48 μ M (MDA MB 157), 0.6 μ M (BT20), 0.67 μ M (SUM159), 0.75 μ M (HS578T) and 0.8 μ M (HCC38) and 1.75 μ M (HCC1395) (**Figure 4C**). Taken together, these data suggests that TNK2-AKT signaling play an important role in TNBC growth and proliferation. Further, TNK2 kinase inhibition by small molecule inhibitor suppresses TNBC proliferation

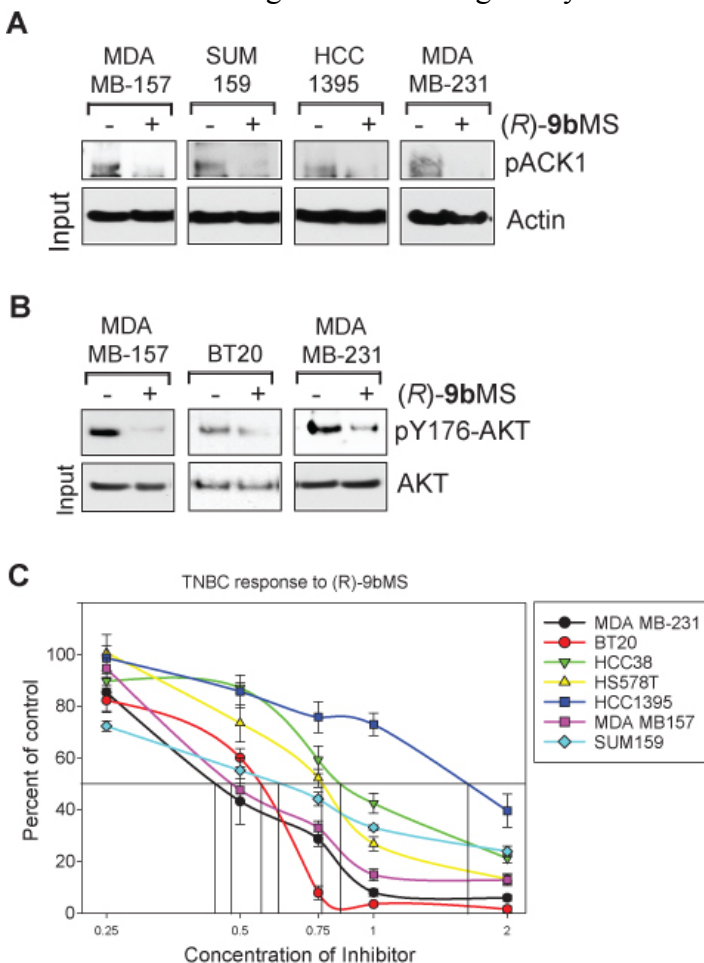


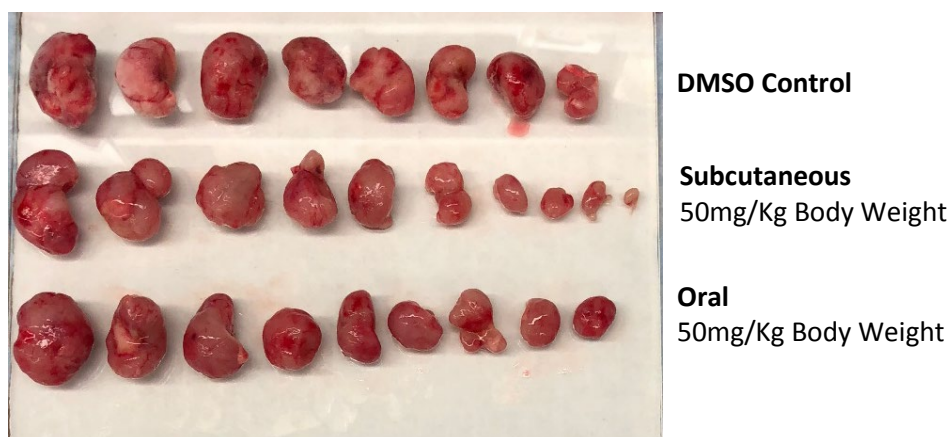
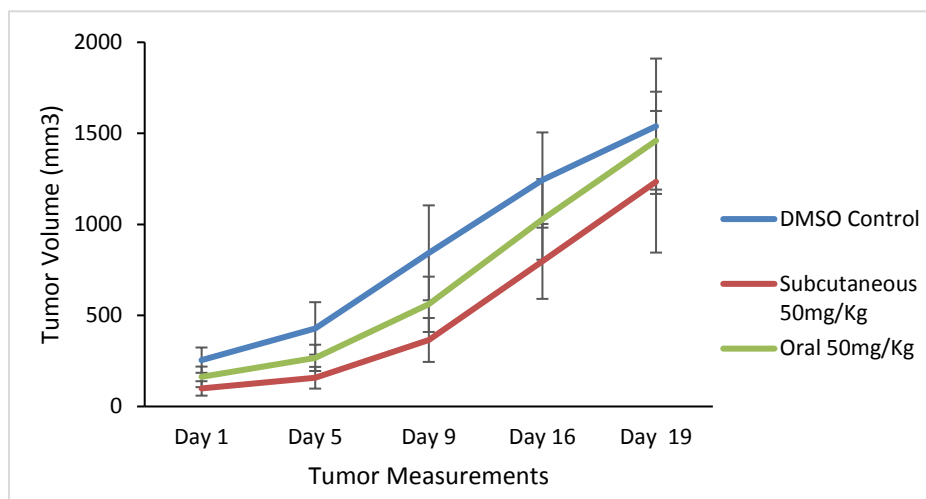
Figure 4. Small molecule inhibitor (*R*)-9bMS sensitizes TNK2 and its substrate AKT kinase phosphorylation compromising TNBC proliferation. (A) TNBCs were treated with TNK2 inhibitor (5 μ M, 24 hours); lysates were immunoprecipitated with ACK1 antibodies, followed by immunoblotting with pTyr-antibodies. (B) TNBC were treated with TNK2 inhibitor (5 μ M, 24 hours); lysates were immunoprecipitated with pY176-AKT antibodies, followed by immunoblotting with pan AKT-antibodies (C) TNK2 inhibitor (*R*)-9bMS overcomes TNBC growth. Cells were treated with (*R*)-9bMS for 96hr and number of viable cells counted by trypan blue exclusion assay. Data are represented as mean $\pm$ SEM ($n = 2$, three replicates).

Major Task 2: To evaluate therapeutic potential of targeting TNK2 in preclinical animal models.

Subtask 2: To perform orthotopic xenograft with TNBC cells and treat with ACK1 inhibitor (R)-9bMS (also known as DZ1-067) when tumors are palpable

Completed: 100%

To test the effect of TNK2 knockdown *in vivo*, we performed xenograft studies on immunocompromised SCID mice. 1×10^6 Cal-148 cells were transplanted into mammary fat pads in mice. Three weeks after the palpable tumors were formed, mice were injected (R)-9bMS subcutaneously (50 mg/Kg) or given oral dose (50 mg/Kg) and tumor growth was measured for 3 weeks.



The mice were euthanized, and tumor were excised (Figure 5). A significant reduction in tumor volume was seen in mice that were injected with (R)-9bMS subcutaneously (table below), suggesting that (R)-9bMS is an excellent therapeutic agent for TNBCs.

Figure 5. Small molecule inhibitor (R)-9bMS inhibit TNBC xenograft tumor proliferation. (Top panel) SCID mice injected with Cal-148 cells followed by inhibitor treatment. Tumor volume was measured. (Bottom panel) Tumors were excised and photographed.

	Day 1	Day 5	Day 9	Day 16	Day 19	t test
DMSO Control	254.476	428.792	843.915	1243.569	1538.948	
Subcutaneous 50mg/Kg	99.18818	157.7582	365.3636	796.7145	1234.05	0.005035
Oral 50mg/Kg	162.7485	267.0031	561.1246	1027.568	1459.861	0.01216

Key Research Accomplishments

1. We have identified a new class of robust and soluble TNK2/ACK1-specific small molecule inhibitor, (R)-9bMS, also known as DZ1-067 that significantly suppressed TNBC proliferation, suggesting that TNBCs are “addicted” to the expression of TNK2 in order to maintain their oncogenic phenotype.
2. Subcutaneous injection of (R)-9bMS significantly compromised TNBC xenograft tumor growth, suggesting TNK2-specific small molecule inhibitor (R)-9bMS as a realistic treatment option for TNBC patients
3. Detection of AKT Tyr176-phosphorylation in TNBCs, its sensitivity to TNK2 inhibitor (R)-9bMS which in turn compromising proliferation of TNBCs indicates that TNK2-AKT signaling may drive TNBC tumor growth.

Reportable Outcomes

Publications:

*Corresponding author

1. Wu X, Zahari MS, Renuse S, Bharbuiya MA, Rojas PL, Stearns V, Gabrielson E, Malla P, Sukumar S, **Mahajan NP*** and **Pandey A***. The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer. *Oncotarget*, 8:2971-2983, 2017. PMID: 27902967; PMCID: PMC5356856
2. Mahajan K, Malla P, Lawrence H, Chen Z, Sinha CK, Malik R, Shukla S, Kim J, Coppola D, Lawrence N and **Mahajan NP***. ACK1 regulates histone H4 Tyr88-phosphorylation and AR gene expression in castration resistant prostate cancer. *Cancer Cell*, 31:790-803, 2017. PMID: 28609657
3. Mahajan K, Malla P, Sharma, V, Sarnaik A, Kim J, Pilon-Thomas S, Weber J and **Mahajan NP***. WEE1 epigenetically modulates 5-hmC levels by pY37-H2B dependent regulation of IDH2 gene expression. *Oncotarget*, 8(63):106352-106368, 2017. PMID: 29290954; PMCID: PMC5739739; DOI: 10.18632/oncotarget.22374

Seminar/Presentations:

- Washington University, St. Louis, MO, 2018. *Epigenetic Targeting of ACK1/TNK2 kinase in Cancer*
- Baylor College of Medicine, Houston, TX, 2017. *Epigenetic Targeting of ACK1/TNK2 kinase in Cancer*
- Sidney Kimmel Cancer Center, Philadelphia, PA, 2017. *Epigenetic Targeting of ACK1/TNK2 kinase in Cancer*
- New Frontiers in Urologic Oncology 2017 Symposium. Clearwater, FL, 2017. *Targeting of ACK1 in Prostate Cancer*
- 5th Florida Prostate Cancer Symposium. Clearwater, FL, 2016. *Epigenetic Targeting of ACK1/TNK2 kinase in Cancer*
- Moffitt Cancer Center, Tampa, September 2015. *WEE1/ACK1 Epigenetic Signaling in TNBCs*
- American Chemical Society, FAME Conference, Exploring (R)-9bMS, a small molecular inhibitor of ACK1-AR epigenetic circuitry in Triple Negative Breast Cancer; May 3 – Saturday, May 5, 2018 (presented by Dr Mithila Sawant, postdoc in Nupam Mahajan lab)

Conclusion

In conclusion, we have identified TNK2/ACK1 tyrosine kinase as an important player in TNBC tumor growth. Further, we have identified a new class of robust and soluble TNK2/ACK1-specific small molecule inhibitor, (*R*)-9bMS, also known as DZ1-067 that significantly suppressed TNBC proliferation, suggesting that TNBCs could be therapeutically targeted by TNK2 inhibitor, (*R*)-9bMS.

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The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer

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ABSTRACT

Breast cancer is the most prevalent cancer in women worldwide. About 15-20% of all breast cancers do not express estrogen receptor, progesterone receptor or HER2 receptor and hence are collectively classified as triple negative breast cancer (TNBC). These tumors are often relatively aggressive when compared to other types of breast cancer, and this issue is compounded by the lack of effective targeted therapy. In our previous phosphoproteomic profiling effort, we identified the non-receptor tyrosine kinase TNK2 as activated in a majority of aggressive TNBC cell lines. In the current study, we show that high expression of TNK2 in breast cancer cell lines correlates with high proliferation, invasion and colony forming ability. We demonstrate that knockdown of TNK2 expression can substantially suppress the invasiveness and proliferation advantage of TNBC cells *in vitro* and tumor formation in xenograft mouse models. Moreover, inhibition of TNK2 with small molecule inhibitor (R)-9bMS significantly compromised TNBC proliferation. Finally, we find that high levels of TNK2 expression in high-grade basal-like breast cancers correlates significantly with poorer patient outcome. Taken together, our study suggests that TNK2 is a novel potential therapeutic target for the treatment of TNBC.

INTRODUCTION

Breast cancer is a heterogeneous disease, with major subtypes categorized by expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor. Triple negative breast cancer (TNBC) is a subgroup of breast cancer cases that lack the expression of all these three receptors. TNBC accounts for ~15% of invasive breast cancers and these cancers are often highly

proliferative, poorly differentiated and associated with poor prognosis [1-4]. Unlike ER positive luminal breast cancers or breast cancers with HER2 amplification that can be treated with endocrine therapy [5] or HER2-targeted therapy [6], no targeted therapy is currently available for patients with TNBCs. A recent study found that several receptor tyrosine kinases including EGFR, MET and c-Kit were transcriptionally upregulated in different subsets of TNBCs [7, 8], suggesting that these upregulated receptor

tyrosine kinases in TNBCs might be potential therapeutic targets for the treatment of TNBCs. However, clinical trials of targeting receptor tyrosine kinases in TNBCs with specific kinase inhibitors have, to date, produced mainly discouraging results. For example, treatment with monoclonal anti-EGFR antibody cetuximab, alone or in combination with cytotoxic chemotherapies, showed minimal improvement in progression-free and overall survival of TNBC patients [9, 10]. These data suggest that molecules other than receptor tyrosine kinases may be involved in driving TNBCs or that one needs to further stratify the patients based on expression of individual molecular markers.

In our previous efforts to uncover the role of tyrosine kinase signaling pathways that are etiologically activated in TNBCs, we used quantitative mass spectrometry-based phosphoproteomics to globally profile the phosphotyrosine proteomes of a panel of 26 TNBC cell lines, leading to identification and quantitation of over 2,500 phosphopeptides [11]. Through systematic phenotypic characterization using invasion and soft agar colony formation assays of each cell line, we found that these TNBC cell lines were highly heterogeneous in terms of their growth rates and invasiveness. By correlating our phosphoproteomic data with oncogenic phenotypes, we identified the non-receptor tyrosine kinase, TNK2, as a protein that was hyperphosphorylated in a majority of aggressive TNBC cell lines, suggesting that TNK2 is a regulator of TNBC growth and proliferation.

TNK2, also known as ACK1 (activated Cdc42-associated kinase), is a non-receptor tyrosine kinase which has been shown to be frequently amplified or mutated in multiple human cancers including breast, esophageal, lung, ovarian, pancreatic and prostate cancer [12, 13]. Overexpression of TNK2 in cancer cell lines can increase the invasive phenotype, both *in vitro* and *in vivo*, resulting in increased mortality of a mouse model of metastasis [13, 14]. Clinically, it was observed that amplification of the *TNK2* gene in primary tumors correlates with poor prognosis [13]. Further, expression of activated TNK2 was positively correlated with the severity of disease progression, and inversely correlated with the survival of breast cancer patients [15]. Being a cytoplasmic kinase, TNK2 couples extracellular signals from multiple receptor tyrosine kinases including EGFR, HER2 and PDGFR to intracellular effectors [15-18].

It has been suggested previously that phosphorylation of TNK2 might correlate with breast cancer progression [15, 19]; however, the functional significance of TNK2 expression and its role in breast cancer biology — particularly in TNBCs — has not been well elucidated. Based on emerging importance of TNK2 in various malignancies, we undertook detailed characterization of this relatively understudied non-receptor tyrosine kinase. In the current study, we show that TNK2 is overexpressed in a majority of TNBC cell lines and TNK2 expression

level is significantly associated with the aggressiveness of TNBCs. By performing genetic ablation studies, we have demonstrated that TNK2 expression not only drives TNBC proliferation but also confers invasiveness, which is reflected in tumor formation in xenograft mouse models. Further, analysis of breast cancer patient survival data revealed that high TNK2 expression is significantly correlated with worse outcome of high-grade TNBC patients. In addition, the importance of TNK2 signaling was also reflected in cell proliferation studies; treatment with TNK2-specific small molecule inhibitor, (*R*)-**9bMS**, significantly suppressed growth of multiple TNBC cell lines. Taken together, these data uncover dependence of TNBCs on TNK2 signaling for both proliferation and invasion capabilities, and offer the promise of TNK2 inhibition as a therapeutic strategy for a subset of aggressive TNBCs.

RESULTS

Phenotype analyses correlate TNK2 levels with cellular aggressiveness

In our previous study characterizing the activated tyrosine signaling pathways of 26 TNBC cell lines [11], we identified four phosphotyrosine sites of TNK2 (pY284, pY518, pY859 and pY860) in multiple TNBC cells lines (Figure 1A) and discovered that TNK2 pY518 is hyperphosphorylated in a majority of aggressive cell lines [11]. In order to examine the expression level of TNK2 in TNBC, we performed immunoblotting for TNK2 in our panel of 26 TNBC cell lines (Figure 1B). A significant fraction (15 out of 26) of TNBC derived cell lines exhibited medium to high levels of TNK2 expression. When we examined how TNK2 expression might be related to anchorage-independent growth and cellular invasion (as determined by colony formation and matrigel invasion assays, described in our previous study [11]), we observed that TNK2 expression levels were significantly correlated with anchorage-independent growth and cellular invasiveness, suggesting that TNK2 plays an important role in regulating oncogenicity of TNBC cells (Figure 1B). Interestingly, HBL100, one of the immortalized non-tumorigenic mammary epithelial cell line, shows a relative aggressive phenotype and high expression of TNK2 in our study. It has been shown that HBL100 cells express the oncogenic SV40 large T antigen [20]. Similar aggressive phenotype of HBL100 has also been reported in other studies [21, 22].

To further test the role of TNK2 in regulating tumorigenicity, we performed siRNA knockdown of TNK2 in four TNBC cell lines with high expression levels of TNK2 (MDA-MB-435, HCC1806, HCC70 and HCC1143) and three cell lines with low levels of TNK2 expression (BT549, HCC1937 and HCC1569).

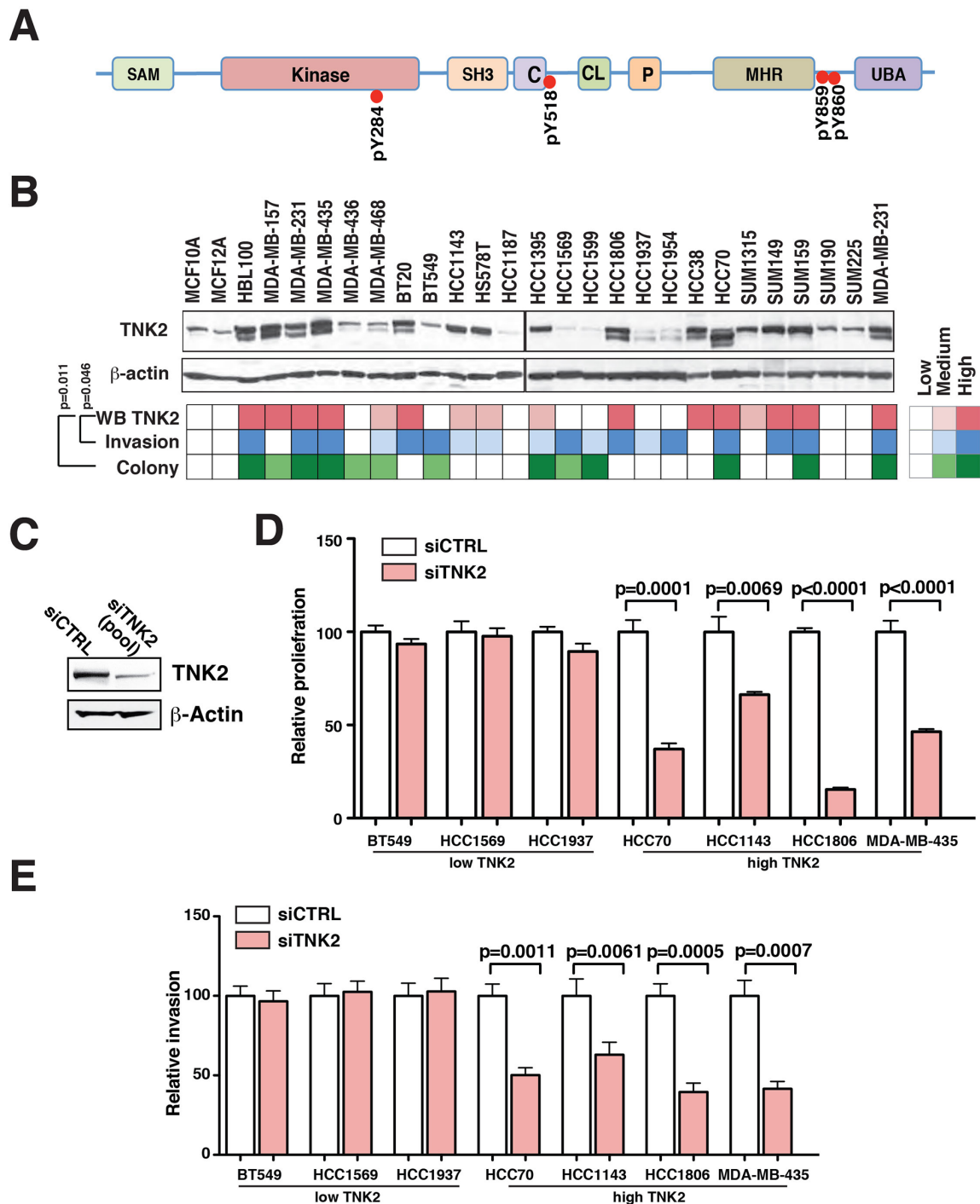


Figure 1: TNK2 is overexpressed in aggressive TNBC cell lines and required for oncogenic phenotype. **A.** A schematic representation of TNK2 domain architecture. Different structural domains present in TNK2 are shown. C, Cdc42-binding domain; CL, clathrin-interacting domain; kinase, tyrosine kinase domain; P, PPXY motif or WW domain-interacting region and SAM, sterile α motif. Tyrosine phosphorylation sites identified in our global phosphoproteomic study are specified. **B.** TNK2 expression level correlates with the aggressive phenotype of TNBC cells. Top panel: Western blot analysis to detect expression of TNK2 in the panel of TNBC cell lines. Color-coded plots showing the expression level of TNK2 (top row), invasiveness (middle row) and colony formation ability (bottom row) across the panel of TNBC cells. Spearman's rank correlation was performed for statistical analysis. **C.** TNK2 siRNA knockdown efficiency was examined in HCC1395 cells. 50 nM siRNA were transfected in HCC1395 cells and an immunoblot was performed to evaluate the knockdown efficiency. β -Actin serves as the loading control. Proliferation **D.** and invasion assay **E.** after knockdown using siRNA against TNK2 (siTNK2) or control siRNA (siCTRL) in TNBC cells that have low expression of TNK2 (BT549, HCC1569, HCC1937) or high expression of TNK2 (HCC70, HCC1143, HCC1806, MDA-MB-435).

The SMARTPool siRNA containing four different siRNA targeting different TNK2 mRNA regions was able to efficiently knockdown TNK2 expression (Figure 1C). We then performed invasion and proliferation assays on these seven manipulated cell lines and found that, in cells expressing high TNK2 levels, proliferation and invasion of cells expressing high TNK2 levels were significantly reduced by knockdown of TNK2. However, the proliferation and invasiveness of the cell lines expressing low levels of TNK2 were not affected with TNK2 knockdown (Figure 1D, 1E and Supplementary Figure 1). These results suggest that high-expression/activation of TNK2 plays an important role in regulating aggressive phenotype of TNBC cells.

TNK2 knockdown reduces aggressiveness of TNBC *in vitro*

Dependence of TNBCs on TNK2 signaling opens up the possibility that genetic ablation of TNK2 in these cells could suppress their proliferation and invasion. To address the role of TNK2 in aggressive breast cancers, we established a lentiviral-based Tet-ON inducible shRNA knockdown system to suppress TNK2 expression in a regulated fashion. The shTNK2 and scrambled control shRNA (shCTRL) expressing lentiviral particles were then used to infect HCC1395 and SUM159 cells—two highly aggressive TNBC cell lines with high basal expression of TNK2. Regulated induction of TNK2 knockdown with doxycycline was determined to be efficacious (Figure 2A, top panel). Upon induction by doxycycline, HCC1395-shTNK2 and SUM159-shTNK2 cells showed significant reduction in colony formation in soft agar (Figure 2B). To calculate cell-doubling time for HCC1395 cells with or without induction of shTNK2, we performed quantitative cell proliferation assays. As shown in Figure 2C, almost no difference in cell proliferation was observed in HCC1395-shCTRL cells regardless of doxycycline induction. However, doxycycline induction of HCC1395-shTNK2 cells substantially prolonged HCC1395 doubling time by >2-fold.

Subsequently, we studied the role of TNK2 on mammosphere formation, which is commonly used to indicate cellular stemness, an important tumorigenic hallmark in breast cancer. We found that upon induction of TNK2 shRNA, the cells completely lost their ability to form mammospheres (Figure 3A). This indicates that TNK2 expression is important for these cells to maintain their stem cell-like properties. To assess the potential effects of targeting TNK2 *in vivo*, we also performed 3-D Matrigel culture assays to better mimic *in vivo* physiological conditions. Similar to our mammosphere assay results, we observed that induction of TNK2 knockdown in HCC1395-shTNK2 cells completely eradicated colonies formed in the matrigel compared to HCC1395-shTNK2 with no induction and to HCC1395-

shCTRL cells (Figure 3B), supporting our hypothesis that TNK2 expression/activation is important for TNBC tumorigenesis *in vivo*.

We then tested whether knocking down TNK2 provided any synergistic activity with doxorubicin, a common chemotherapy agent. We observed that cells with TNK2 knock down by shRNA became rounder and started to die at a much lower dose of doxorubicin compared to cells that were treated with the same concentration of doxorubicin but were not induced to express TNK2 shRNA, which showed a more normal morphology (Figure 3C). To determine if cells were undergoing apoptosis due to these treatments, we performed western blots to examine the apoptosis markers including the cleaved poly(ADP-ribose) polymerase 1 (PARP) and Caspase 8. As shown in Figure 3D, the levels of cleaved PARP and Caspase 8 were substantially increased in shTNK2 knockdown cells treated with doxorubicin compared to the cells without shTNK2 induction. Taken together, these results suggest a synergistic effect in reducing TNK2 expression with the treatment of chemotherapy agents such as doxorubicin.

TNK2 knockdown reduces aggressiveness of TNBC *in vivo*

To test the role of TNK2 in tumor growth *in vivo*, we established orthotopic xenograft using Tet-ON shTNK2 or shCTRL HCC1395 cells transplanted into mammary fat pads of NOD-SCID mice. shRNA (shTNK2 or shCTRL) expression was induced *in vivo* by doxycycline dissolved in drinking water. As shown in Figure 4A, tumor size in the doxycycline-induced shTNK2 group was significantly reduced as compared to the control group (Figure 4A, 4B). Western blot analysis for TNK2 protein expression confirmed the decrease in TNK2 expression in doxycycline-induced shTNK2 xenograft tumors (Figure 4C). Histopathological examination of the non-induced shTNK2 tumors demonstrated a lack of well-defined capsular margins, with cells pushing the borders for invasion compared to the shTNK2-induced tumors (Figure 4D). Immunohistochemical examination of tumor sections with Ki67, a marker of proliferation, and CD31, a marker of vascular endothelial cells, revealed that the shTNK2-induced tumors indeed showed significantly lower extent of proliferation and vascularization (Figure 4D). Overall, our data shows that TNK2 plays important roles in conferring oncogenic phenotype in aggressive TNBCs *in vivo*.

TNK2 levels correlate with poor outcome in patients

To address the clinical relevance of TNK2 expression, we first examined the TNK2 expression levels in TCGA dataset using Oncomine concept analysis. As shown in Figure 5A, TNK2 mRNA expression level is

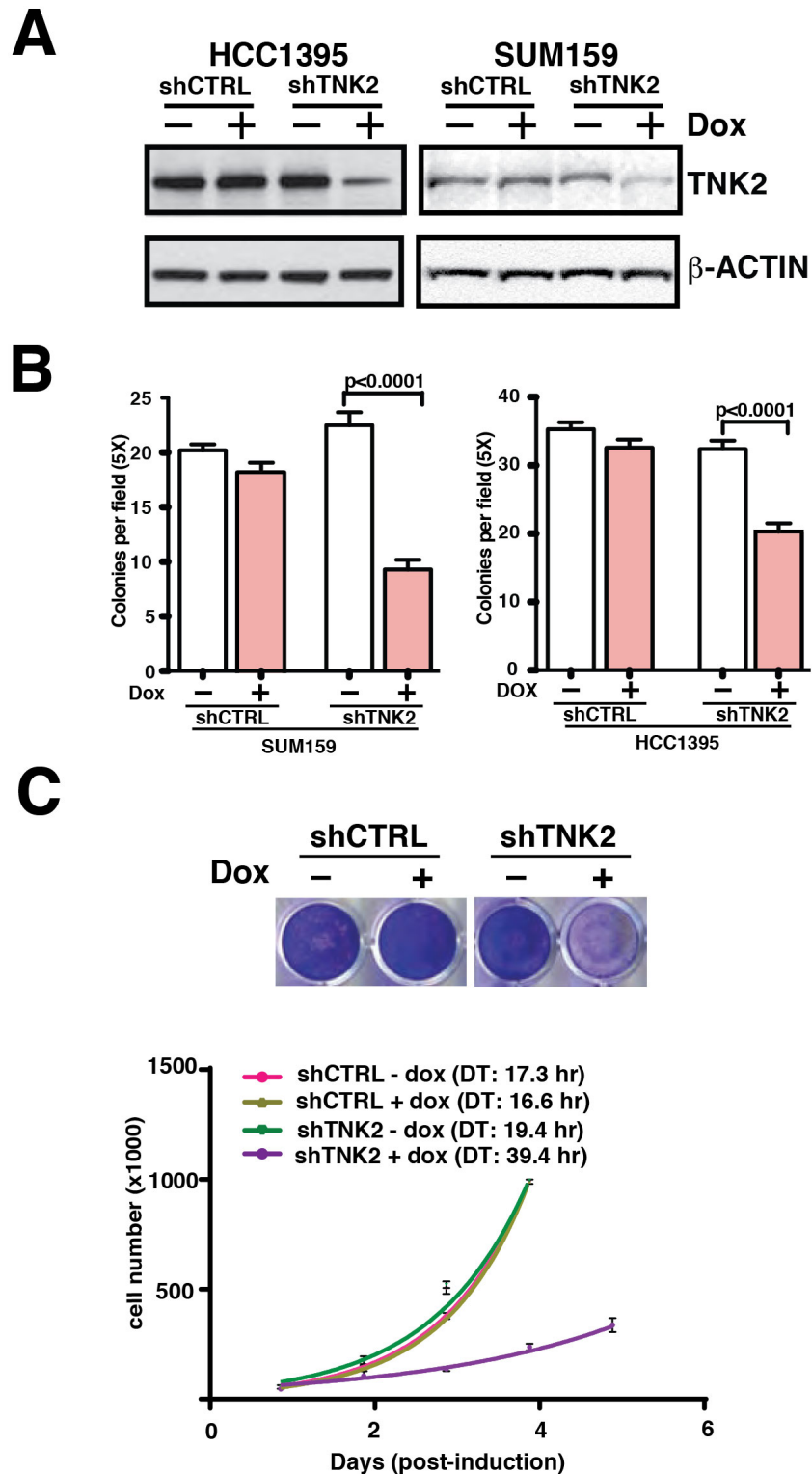


Figure 2: Inducible shRNA knockdown of TNK2 suppresses TNBC oncogenic phenotypes *in vitro*. **A.** Western blot analysis to examine TNK2 expression in HCC1395 and SUM159 cells with inducible TNK2 shRNA or scrambled control shRNA. 100 ng/ml doxycycline was used for induction. **B.** Colony formation assays with shTNK2/shCTRL transfected SUM159 and HCC1395 cells with or without doxycycline induction. Mann-Whitney tests were performed for statistical analyses. **C.** Cell proliferation assays with shTNK2 and shCTRL HCC1395 transfected cells with or without doxycycline induction. The cells were stained with crystal violet to visualize the difference in the number of cells (top) or the doubling time (DT) calculated for each condition (bottom).

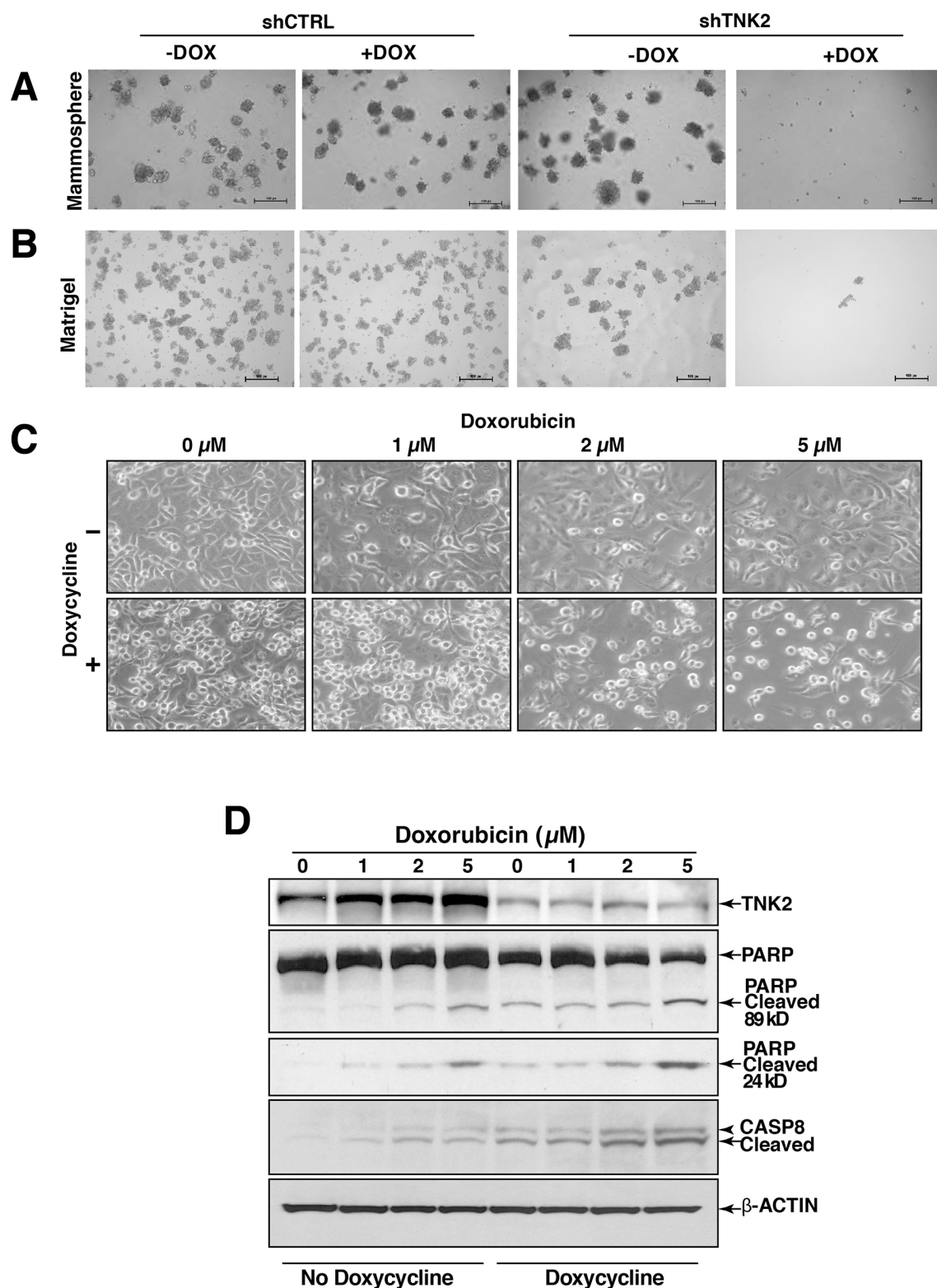


Figure 3: TNK2 is required for mammosphere formation. Mammosphere assays **A.** and 3D matrigel assays **B.** of shTNK2/shCTRL HCC1395 cells with or without doxycycline induction. **C.** Synergistic effect of knocking down TNK2 with doxorubicin treatment. Doxycycline was used to induce shTNK2 expression. Induced or uninduced cells were treated with different doses of doxorubicin (as indicated). **D.** Western blot analysis to examine cleaved PARP and Caspase 8 in shCTRL and shTNK2 cells treated with doxorubicin and with or without doxycycline induction of shRNA. β -Actin serves as a loading control.

lowest in normal breast and highest in TNBC comparing to other ER+ luminal and HER+ breast cancers. We also performed survival analysis using a publicly available gene expression database of breast tumors from 4,142 patients [23]. With our analysis focusing on the 669 tumors that were classified as basal-like breast cancers, we found no significant correlation between TNK2 expression and patient survival. However, when a subpopulation of 237 high grade basal-like breast cancer tumors were selected for the analysis, high TNK2 expression levels were significantly associated with poorer patient outcomes ($p < 0.01$) (Figure 5B). It is also worth noting that TNK2 expression did not show a statistically significant correlation with clinical outcomes for patients with high grade ER-positive tumors (Figure 5C, 5D). We believe this clinical result when added to the results from our *in vitro* and *in vivo* studies provides strong evidence to support identifying TNK2 as a novel target for personalized therapy for patients with basal-like/triple negative breast cancers that express high levels of this protein kinase.

TNK2 kinase inhibition by small molecule inhibitor suppresses TNBC proliferation

Ability of TNK2 to directly phosphorylate AKT at Tyr176 site leading to its activation [15] indicates that TNK2-AKT signalling nexus may be involved in driving

TNBC tumorigenicity. Recently, we have reported a new class of robust and soluble TNK2-specific small molecule inhibitor, (*R*)-**9bMS** [24, 25]. To address the role of TNK2 in AKT Tyr176-phosphorylation in TNBCs, cells were treated with (*R*)-**9bMS**. A significant decrease in TNK2 activation, as seen by loss of TNK2 Tyr-phosphorylation is observed upon (*R*)-**9bMS** treatment (Figure 6A). Inhibition of TNK2 activation is also reflected in significant decrease in AKT Tyr176-phosphorylation (Figure 6B). Further, several TNBC cell lines were found to be sensitive to the (*R*)-**9bMS** treatment with IC_{50} of 0.45 μ M (MDA-MB-231), 0.48 μ M (MDA MB 157), 0.6 μ M (BT20), 0.67 μ M (SUM159), 0.75 μ M (HS578T) and 0.8 μ M (HCC38) and 1.75 μ M (HCC1395) (Figure 6C). Taken together, these data suggest that TNK2-AKT signaling may play an important role in TNBC growth and proliferation.

DISCUSSION

In our previous study, we investigated the intrinsic heterogeneous signaling networks present within the TNBC cell population [11]. In this study, as an extension to our previous work, we focused on a non-receptor tyrosine kinase, TNK2, which is an important signaling molecule involved in integrating signals from a number of different receptor tyrosine kinases [12]. Although TNK2 has also been reported to be amplified and mutated in

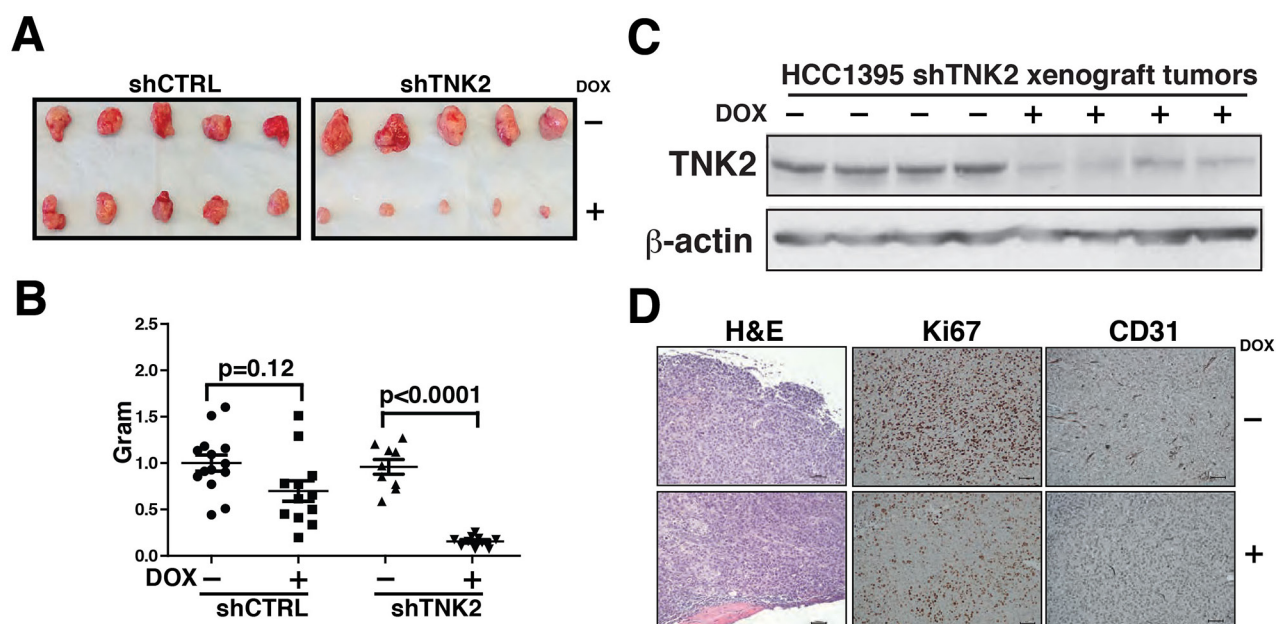


Figure 4: TNK2 knockdown suppresses tumor formation. **A.** Tumors harvested from mice injected with HCC1395-shTNK2 cells or HCC1395-shCTRL cells with or without doxycycline induction. **B.** Weight of tumors resected from mice xenografted with HCC1395-shCTRL or HCC1395-shTNK2 cells with or without doxycycline induction. P-values from Mann-Whitney tests to measure the statistical significance of size differences across the indicated groups are shown. **C.** Expression of TNK2 or beta actin detected by western blotting using specific antibodies in HCC1395 xenografted tumors is shown for the various tumors. **D.** Hematoxylin and eosin staining or immunohistochemical labeling for Ki67 and CD31 in HCC1395- shTNK2 xenografted tumors with (+) or without (-) induction of shTNK.

multiple cancers [12], in breast cancer, its activation is likely to be facilitated by receptor tyrosine kinases that correlate with disease progression as well as survival.

Our global phosphotyrosine study identified several tyrosine phosphorylation sites in TNK2 (Figure 1A) in a number of TNBC cell lines and phosphorylation level of pY518 is elevated in TNBC cells with aggressive phenotypes [11]. The current study has revealed that TNK2 is not only hyperphosphorylated, but also overexpressed in many highly aggressive human TNBC tumors and also in TNBC cell lines, and that the expression of TNK2 correlates with aggressive phenotypes of TNBC cell lines. We also demonstrated

that targeting TNK2 with specific siRNA/shRNA can substantially attenuate oncogenic phenotypes of TNBC cells *in vitro* and, more importantly, dramatically reduce tumor formation in preclinical xenograft mouse models, suggesting that these aggressive characteristics are driven by TNK2. Finally, our study shows promise for combining anti-TNK2 therapy with doxorubicin treatment for aggressive TNBCs, providing a pathway for future clinical applications of our findings.

Recent studies have shown that several ubiquitin ligases including NEDD4-1 [26], NEDD4-2 [27], SIAH1 and SIAH2 [28] can ubiquitinate TNK2 and induce TNK2 degradation. Of note, in ER positive breast cancer cells,

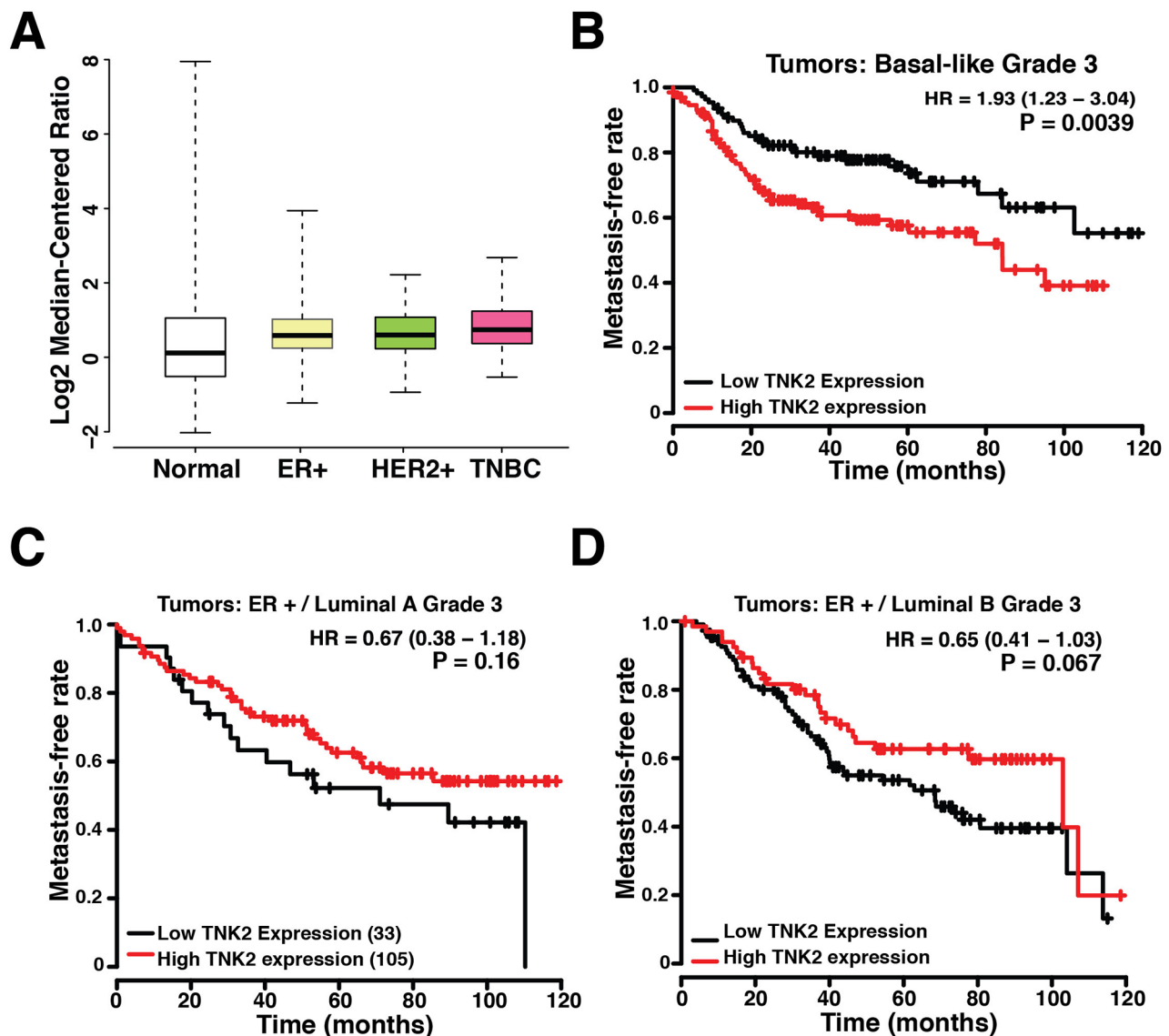
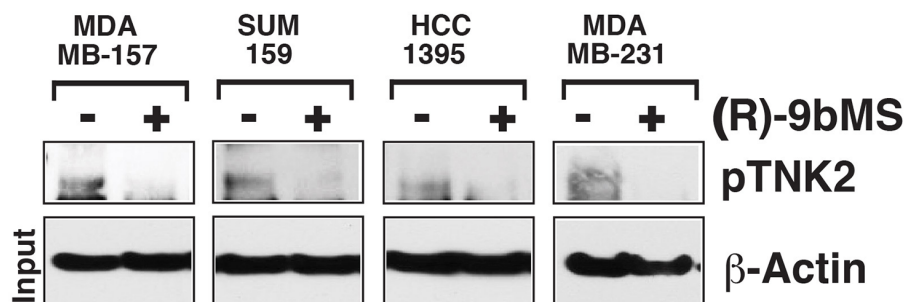
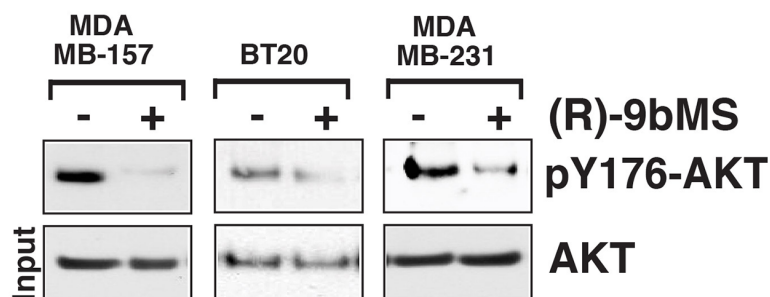


Figure 5: TNK2 levels correlate with poor outcome in patients. A. TNK2 expression level in normal breast and different subtypes of breast cancer. Expression data were retrieved from Oncomine using TCGA dataset and re-plotted with R package. B. Kaplan-Meier plot of 237 high-grade basal-like breast cancer patients stratified with high or low *TNK2* gene expression. C-D. Kaplan-Meier plots of 138 high-grade luminal A (C) and 184 luminal B (D) breast cancer patients. The red line represents the survival curve of patients with high expression of TNK2 and the black line represents the surviving curve of patients with low expression of TNK2.

A



B



C

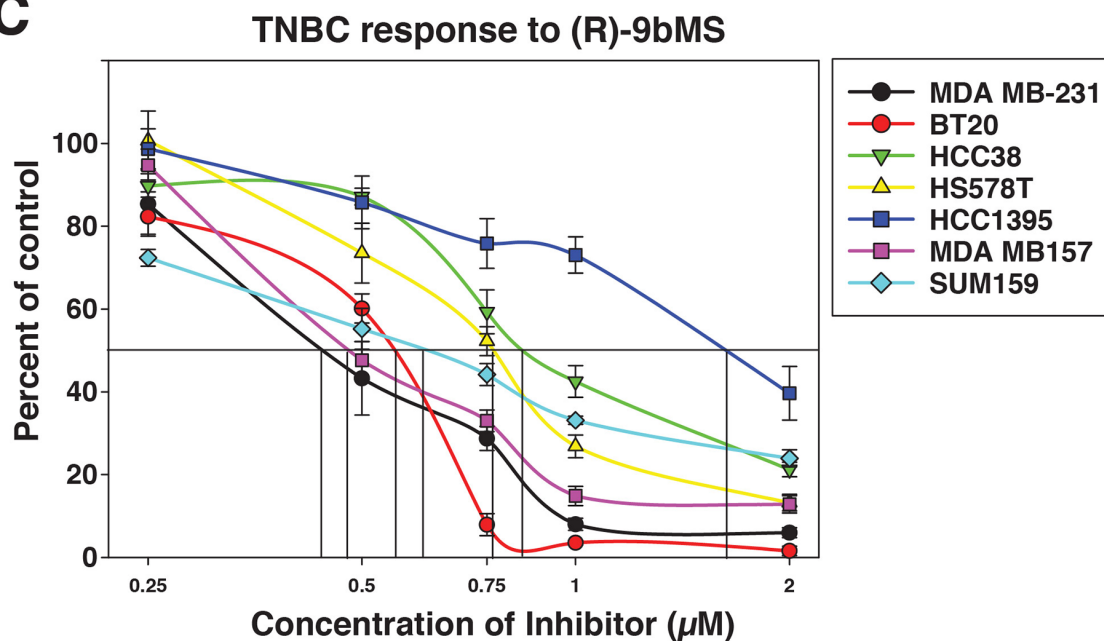


Figure 6: Small molecule inhibitor (R)-9bMS sensitizes TNK2 and its substrate AKT kinase phosphorylation compromising TNBC proliferation. **A.** TNBCs were treated with TNK2 inhibitor (5 μ M, 24 hours); lysates were immunoprecipitated with TNK2 antibodies, followed by immunoblotting with pTyr-antibodies. **B.** TNBC were treated with TNK2 inhibitor (5 μ M, 24 hours); lysates were immunoprecipitated with pY176-AKT antibodies, followed by immunoblotting with pan AKT-antibodies. **C.** TNK2 inhibitor (R)-9bMS reduces TNBC growth. Cells were treated with (R)-9bMS for 96hr and the number of viable cells were counted by trypan blue exclusion assay. Data represented as mean $\pm$ SEM ($n = 2$, three replicates).

estrogen can activate ER and induce SIAH2 expression that subsequently ubiquitinates TNK2 and reduces TNK2 expression. Absence of ER in TNBC could be a potential reason why TNK2 is more expressed in TNBC (Figure 5A). A major downstream effector of TNK2 in hormonally regulated cancers has been AR [29], which also points to the potential of ER as another TNK2-interacting entity. However, TNBCs present a conundrum as although these cells lack expression of ER, they are exquisitely sensitive to loss of TNK2, suggesting that some other downstream effectors may be operational in TNBCs. TNK2 has been shown to interact with and directly phosphorylate AKT at an evolutionarily conserved tyrosine 176 residue, promoting AKT activation. Detection of AKT Tyr176-phosphorylation in TNBCs (Figure 6A), its sensitivity to TNK2 inhibitor (*R*)-**9bMS** which in turn compromising proliferation of TNBCs (Figure 6B) indicate that TNK2-AKT signaling may drive TNBC tumor growth. Future studies may reveal whether sensitization of TNBCs by TNK2 inhibitor also involves other TNK2 effectors.

About 10-20% of breast cancers are found to be triple-negative. Due to limited treatment options that are currently available, there is intense interest in finding new therapies that can treat this subtype of breast cancer. This study not only provides evidence for ‘addiction’ of TNBCs to TNK2 signaling, but also provides a rationale for exploring TNK2-specific small molecule inhibitor (*R*)-**9bMS** as a realistic treatment option for TNBC patients.

MATERIALS AND METHODS

Cell culture

Cell lines were obtained from ATCC and cultured in the appropriate media as previously described [11, 30, 31].

Western blot and siRNA knockdown

Each cell line was harvested and lysed in modified RIPA, and immunoblotting was performed as previously described [32]. The primary antibodies used in this study are anti-TNK2 (Santa Cruz Biotechnology), anti-PARP cleaved 89 Kd protein (Cell Signaling), anti-PARP cleaved 24 kD protein (Abcam), anti-cleaved Caspase 8 (Cell Signaling) anti-AKT (Cell Signaling), and anti-pY176-AKT antibody that was developed in our previous study [15]. 50 nM siRNA targeting TNK2 (L-003102, SMARTpool, Dharmacon) was used for transfections with RNAiMax (Invitrogen). Cells were harvested 48 hours post-transfection for assessing knockdown efficiency or other follow-up experiments.

Matrigel invasion assays

Cells were washed once with PBS, detached using trypsin (Life Technologies) and 5×10^4 cells were seeded into Biocoat matrigel invasion chambers (BD Biosciences). Growth media supplemented with serum

for each cell line was added in the lower wells as the chemoattractant. After 24 hours, the filter membranes were stained with DAPI (Invitrogen). The number of cells that penetrated through the matrigel and membrane was counted for ten randomly selected viewing fields at 20x magnification. ImageJ was used to count cell nuclei. Student's t-test was performed for statistical analysis.

Soft agar colony formation assays

Briefly, 0.5 ml of 0.8% bottom layer agar was prepared in six-well plates. Cells with different treatments were separately trypsinized, centrifuged, resuspended in 0.4% agar medium (equal volumes of 0.8% agar and culture medium), and plated onto the top agar at 500 cells per well. The cells were grown for 14 days at 37°C. Colonies were then stained with crystal violet and counted under the microscope. Mann-Whitney tests were performed for statistical analyses.

MTT cell proliferation assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assays were performed to measure cell proliferation. Briefly, cells that were transfected with different siRNAs in 96-well plate were left to grow for 5-7 days before the MTT assay. 1 mg/ml MTT in growth media was added into each well and the plate was incubated for two hours in 37°C. Media was then removed and 100 μ l of DMSO and ethanol (1:1 by volume) was added into each well. The plate was then read for absorption at 530 nm on a microplate reader. Student's t-test was performed for statistical analysis.

To determine effect of TNK2 inhibitor, cells were treated with different concentration of (*R*)-**9bMS** for 96 hours and live cells (determined by trypan blue exclusion) were counted.

Generation of inducible shTNK2 cells

A shRNA construct targeting a different TNK2 sequence (GGCAGUCAGAUCCUGCAUAAG) from that targeted by siRNA was designed and cloned into the pLKO-Tet-On-puro plasmid vector (Addgene) and the sequence was confirmed by Sanger sequencing. The plasmid was then packaged into viral particles by transfection into HEK293T cells along with packaging plasmids, psPAX2 and pMD2.G. After overnight transfection, the conditioned medium was collected and used to infect SUM159 and HCC1395 cells. Stably infected cells were selected by puromycin.

In vivo tumor xenograft assays

NOD-SCID mouse xenografts were performed as previously reported [33]. Briefly, 20 four to six week old female NOD-SCID mice were purchased from NCI. HCC1395 cells stably infected with Tet-ON inducible

TNK2 shRNA or control shRNA were resuspended in matrigel:PBS (1:1 volume). 5×10^5 cells were injected onto the mammary fat pads of NOD-SCID mice at two sites per mouse. Mice were then either administered doxycycline in drinking water (2 mg/ml) to induce the expression of shRNA or normal water as control. Five mice were used for each treatment group. After four weeks, the mice were sacrificed and tumors were processed. Student's t test was performed for the statistical analysis to compare the tumor weight. All procedures were approved by Johns Hopkins University institutional Animal Care and Use Committee and were performed in accordance with the Animal Welfare Act regulations.

Immunohistochemical labeling of xenograft tumors

Immunohistochemical staining was performed on the formalin-fixed, paraffin-embedded xenograft tumors. Briefly, paraffin embedded slides were rehydrated and then treated with hydrogen peroxide to block endogenous peroxidase activity, and then washed with PBST buffer. Slides were then blocked with goat serum before diluted rabbit polyclonal antibodies to Ki67 and CD31 were added for protein binding at 4°C and incubated overnight. The slides were washed with PBST, incubated with biotinylated secondary antibody (Dako), and treated with DAB substrate kit (Dako) according to the manufacturer's instructions. The slides were then counter-stained using Mayer's Hematoxylin (Dako) before visualizing under the microscope.

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CONFLICTS OF INTEREST

N.P.M. is named as inventors on a patent application no. 60/862,763 and 62/200,084 titled 'Inhibitors of ACK1/TNK2 Tyrosine Kinase'.

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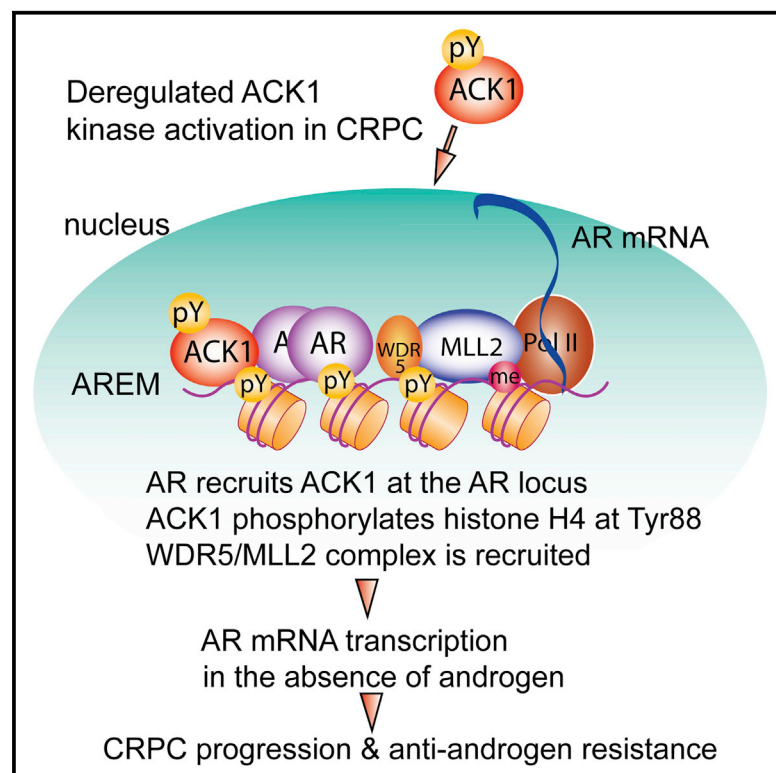
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ACK1/TNK2 Regulates Histone H4 Tyr88-phosphorylation and AR Gene Expression in Castration-Resistant Prostate Cancer

Graphical Abstract



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In Brief

Mahajan et al. report that ACK1 phosphorylates histone H4 at Y88 upstream of the *AR* transcription start site, leading to the WDR5/MLL2 complex-mediated increase of *AR* transcription. Inhibition of ACK1 reverses the pY88-H4 marks and reduces AR and AR-V7 levels to mitigate castration-resistant prostate tumor growth.

Highlights

- Characterization of a histone H4-Y88 phosphorylation in CRPCs
- Androgen receptor is a key epigenetic target of the H4-Y88 phosphorylation in CRPCs
- Targeting H4-Y88 phosphorylation subdues the expression of AR and AR splice variant
- The epigenetic inhibitor (*R*)-9bMS mitigates enzalutamide resistance



ACK1/TNK2 Regulates Histone H4 Tyr88-phosphorylation and AR Gene Expression in Castration-Resistant Prostate Cancer

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SUMMARY

The androgen receptor (AR) is critical for the progression of prostate cancer to a castration-resistant (CRPC) state. AR antagonists are ineffective due to their inability to repress the expression of AR or its splice variant, AR-V7. Here, we report that the tyrosine kinase ACK1 (TNK2) phosphorylates histone H4 at tyrosine 88 upstream of the AR transcription start site. The WDR5/MLL2 complex reads the H4-Y88-phosphorylation marks and deposits the transcriptionally activating H3K4-trimethyl marks promoting AR transcription. Reversal of the pY88-H4 epigenetic marks by the ACK1 inhibitor (R)-9bMS-sensitized naive and enzalutamide-resistant prostate cancer cells and reduced AR and AR-V7 levels to mitigate CRPC tumor growth. Thus, a feedforward ACK1/pY88-H4/WDR5/MLL2/AR epigenetic circuit drives CRPC and is necessary for maintenance of the malignant state.

INTRODUCTION

Androgen receptor (AR) plays a paramount role in the onset and progression of prostate cancer (PC) (Burnstein, 2005; Lonergan and Tindall, 2011; Watson et al., 2015). This very facet underlies androgen deprivation therapy (ADT), a preferred treatment to negate AR transcriptional activity. While ADT provides immediate palliative benefits, it is ineffective long term, as the recalcitrant disease recurs within 2–3 years and progresses to a lethal stage, referred to as the metastatic castration-resistant prostate cancer (mCRPC). The AR gene (AR) is amplified or mutated

in >50% of mCRPCs (Grasso et al., 2012; Robinson et al., 2015), suggesting that PC cells may have reinvigorated AR transcription as a response to the loss of existing AR activity by ADT. Consequently, resistance to ADT has become one of the most vexing problems in PC therapy.

CRPC cells rely on AR for their growth despite androgen depletion; not surprisingly, AR has been the epicenter of targeted therapies. However, while enzalutamide, a second-generation AR antagonist, efficiently antagonized AR transcriptional activity by overcoming its nuclear translocation (Tran et al., 2009), the overall survival advantage was found to be ~6 months, and

Significance

Castration-resistant prostate cancer (CRPC) defines the incurable stage of disease. While the second-generation anti-androgen enzalutamide and the androgen-synthesis inhibitor abiraterone stall CRPC progression for a limited time, tumors inevitably develop resistance by replenishing either the androgen receptor (AR) or its spliced variant AR-V7, which lack the ligand-binding region. We uncovered ACK1 kinase as an epigenetic modifier hijacked by AR to modify its own gene locus, reactivating AR mRNA synthesis. Significantly, targeting this epigenetic deregulation with the ACK1 inhibitor (R)-9bMS not only suppressed AR and AR-V7 transcription but also overcame enzalutamide resistance and mitigated CRPC tumor growth. This study demonstrates an epigenetic mechanism of tyrosine kinase-driven CRPC growth and suggests a therapeutic strategy.

most patients relapsed within 2 years (Bennett and Ingason, 2014). Interestingly, these relapsed patients exhibit renewed AR-regulated gene expression by multiple mechanisms, suggesting that CRPCs overcome enzalutamide blockade (Arora et al., 2013; Balbas et al., 2013; Joseph et al., 2013; Korpál et al., 2013). The AR splice variant-7 (AR-V7) is a truncated form of AR that lacks the C-terminal ligand-binding domain and remains constitutively active as a transcription factor (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009; Lu et al., 2015). Recent studies suggest that AR-V7 may be a clinically relevant mechanism of resistance to enzalutamide and the androgen-synthesis inhibitor abiraterone in CRPC patients (Antonarakis et al., 2014). The relative short-term efficacy of enzalutamide and abiraterone reveals two major caveats for tackling this complex disease; first, not all CRPCs are the same; and, second, other signaling events may be driving the disease. Moreover, because CRPCs display *de novo* or intrinsic ability to increase AR levels, inhibition of AR protein activity is not enough. To achieve complete remission, ablation of AR appears to be the key. However, targeted inhibition of transcription of AR and AR-V7 with small-molecule inhibitors has not yet been accomplished.

Resistance to ADT is closely associated with abnormal tyrosine kinase signaling; non-receptor tyrosine kinases (NRTKs), such as ACK1 and SRC, are known to interact with AR in an androgen-independent manner to promote CRPC xenograft growth (Guo et al., 2006; Mahajan and Mahajan, 2010; Mahajan et al., 2007). ACK1 is a structurally unique NRTK upregulated in ~25% of prostate adenocarcinomas (Mahajan et al., 2010b; Mahajan and Mahajan, 2015; Taylor et al., 2010). Importantly, 10 out of 13 CRPCs exhibited 5- to >100-fold ACK1 overexpression (van der Horst et al., 2005). Further, LNCaP cells that are poorly tumorigenic in castrated mice formed robust CRPC tumors following expression of activated ACK1 (Mahajan et al., 2005). Moreover, the expression of activated ACK1 correlates positively with the progression of disease to CRPC stage, and PC patients whose tumors display moderate to strong staining of activated ACK1 have poor prognosis (Mahajan et al., 2010a). Combined, these studies have established a crucial role for ACK1 in PC pathogenesis. In this study, we investigated whether ACK1 tyrosine kinase promotes chromatin alterations to drive CRPC progression.

RESULTS

Identification of Tyr88-Phosphorylated Histone H4 in Human CRPCs

Epigenetic alterations have emerged to be an underlying mechanism in CRPC pathogenesis (Grasso et al., 2012). To examine a potential role for an epigenetic alteration/s in CRPCs, histones were purified from five freshly frozen human CRPCs and subjected to mass spectrometry-based identification of post-translational modifications. This unbiased approach led to the identification of phosphorylation of tyrosine 88 in histone H4 in 3 out of 5 CRPC biospecimens (Figures S1A and S1B). The Y88-phosphorylation of H4 in a human CRPC sample was also assessed by immunoblotting; compared with a normal prostate sample, robust H4 Y88-phosphorylation was detected in the CRPC sample (Figure S1C). Notably, Tyr88 in histone H4 is evolutionarily

conserved, suggesting an important physiological function (Figure S1D). As the functional role of Tyr88-phosphorylated H4 (pY88-H4) is unknown, we generated a high-affinity monoclonal antibody against pY88-H4.

The pY88-H4 antibody specifically recognized the Tyr88-phosphorylated H4 peptide but failed to recognize the unphosphorylated peptide, and the phosphopeptide competed with pY88-H4 antibody for binding, dampening the signal (Figure S2A). Moreover, pY88-H4 antibody was screened for cross-reactivity against 59 acetylation, methylation, phosphorylation, and citrullination modifications of histones using the histone peptide array, as described in an earlier publication (Mahajan et al., 2012b). The pY88-H4 antibody did not cross-react with any of these histone modifications, whereas the H3K4me3 antibody revealed the expected pattern of hybridization (Figure S2B). Phosphorylation of H4 at Tyr88 was also detected by direct immunoblotting (Figure S2C) as well as by immunofluorescence; pY88-H4 staining was predominantly seen in the nuclei of the ACK1 expressing cells (Figure S2D).

To determine the tyrosine kinase modifying H4 at Y88, total histones were purified from activated ACK1- or SRC-expressing cells, followed by mass spectrometry. The phosphorylation of H4 at Y88 was detected only in histones isolated from the activated ACK1-expressing cells (Figures S2E–S2G). To confirm the kinase responsible for the H4 phosphorylation, HEK293T cells were transfected with MYC-tagged H4, followed by pull-down using MYC-beads and mass spectrometry. This revealed the presence of ACK1, which was phosphorylated at Tyr 827 (Figures S2H and S2I), suggesting that activated ACK1 is a major kinase that binds to H4. In addition to ACK1, many other well-known nuclear proteins, e.g., PARP, RNA helicase DHX9, XRCC5, XRCC6, DAXX, MSH2, MRE11, and histone acetyltransferase HAT1, were also identified in this proteomics approach (Table S1).

To further assess the role of ACK1 as a bona fide histone kinase *in vitro* and *in vivo*, we treated PC cells with an ACK1 small-molecule inhibitor, (R)-9bMS (Figure 1A and S3A–S3C; Table S2). (R)-9bMS is a mesylate salt derivative of (R)-9b that we developed and characterized as an ACK1 inhibitor (half maximal inhibitory concentration [IC₅₀] = 48 nM) with considerable stability in human plasma and a long half-life (Lawrence et al., 2015). (R)-9bMS is significantly more soluble in aqueous medium than the parent compound, thus making it an attractive candidate for *in vivo* studies.

To uncover any other kinases that may be potentially targeted by (R)-9bMS, we performed specificity profiling, wherein its activity against a large panel of kinases (n = 369) at 250 nM was assessed. Apart from ACK1, only 11 other kinases were inhibited *in vitro* to >90% (Table S2 and Figure S3C). The Human Protein Atlas reveals that 8 of these 11 kinases, TNK1, LRRK2, JAK1–3, TYK2, and NUA1 and NUA2, are either not expressed or expressed at very low levels in prostate tissues and cancers. The remaining three kinases, FLT3, PKCmu, and SIK2, have not yet been shown to be critical for CRPCs. Thus, while *in vitro* studies indicate other potential targets, at least in CRPCs, ACK1 appears to be the predominant target of (R)-9bMS.

To assess the direct binding of ACK1 to histone H4, an *in vitro* kinase assay was performed with purified proteins. Purified recombinant human ACK1 protein was incubated with purified

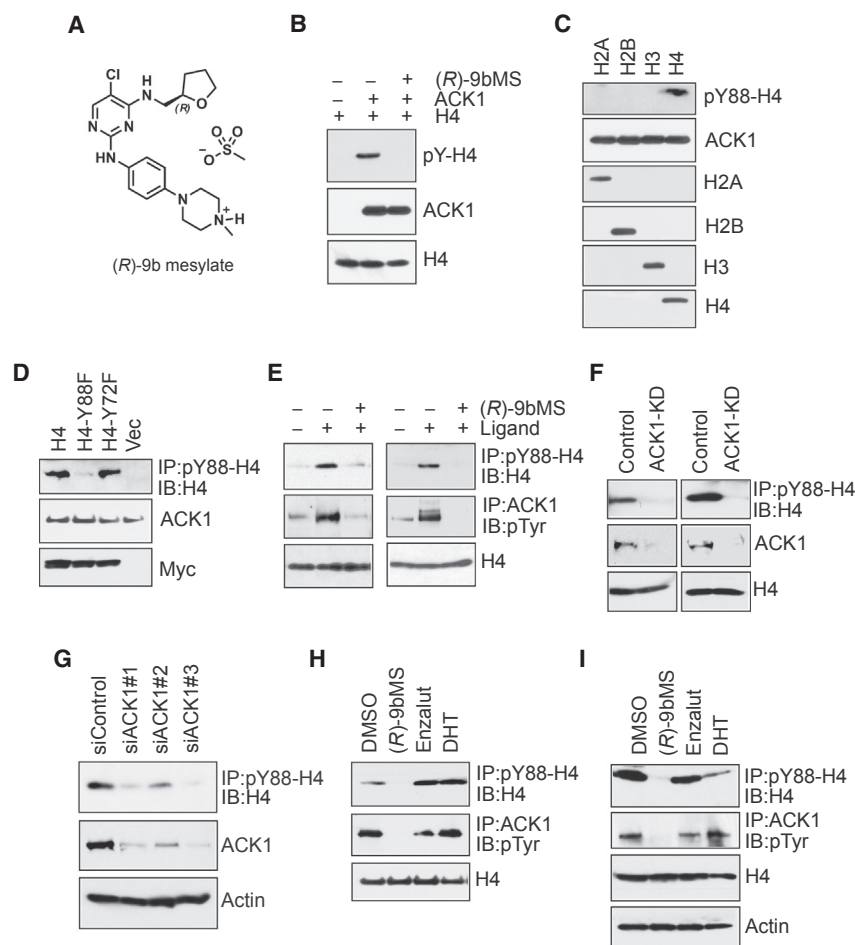


Figure 1. Androgen-Independent Phosphorylation of Histone H4 at Tyrosine 88 by ACK1
(A) Chemical structure of (R)-9b mesylate salt or (R)-9bMS.

(B) Purified histone H4 alone or incubated with equimolar of purified ACK1 in the absence or presence of 100 nM (R)-9bMS then subjected to immunoblotting using the indicated antibodies.

(C) In vitro kinase assay performed using purified ACK1 and indicated histones, followed by immunoblotting with indicated antibodies.

(D) HEK293T cells co-transfected with ACK1 and MYC-tagged H4 or mutants, followed by immunoblotting.

(E) Serum-starved LNCaP (left) and LAPC4 (right) cells were treated with (R)-9bMS overnight followed by stimulation with IGF for 20 min or platelet-derived growth factor for 60 min, respectively. Lysates were immunoprecipitated with pY88-H4 or ACK1 antibodies followed by immunoblotting with H4 or pTyr antibodies, respectively.

(F) WT C4-2B cells or two distinct clones of C4-2B cells lacking ACK1 generated using the CRISPR-Cas9 gene editing system were serum starved and treated with IGF for 20 min. Lysates were immunoprecipitated with pY88-H4 antibody followed by immunoblotting with H4 antibody.

(G) Androgen-deprived LNCaP cells were electroporated with control or three distinct ACK1 siRNAs, followed by treatment with IGF for 20 min. Lysates were immunoprecipitated with pY88-H4 antibody followed by immunoblotting with H4 antibody. Lysates were also subjected to immunoblotting with ACK1 antibody.

(H and I) Serum- and androgen-starved C4-2B (H) and LNCaP (I) cells were treated with IGF for 20 min, and lysates were immunoprecipitated

with pY88-H4 or ACK1 antibodies, followed by immunoblotting with H4 or pTyr antibodies, respectively. See also [Figures S1–S3](#), and [Tables S1 and S2](#).

H4 followed by immunoblotting with pTyr antibody. H4 Tyr-phosphorylation was abrogated upon (R)-9bMS treatment ([Figure 1B](#)). Further, purified ACK1 was incubated with all the four core histones, followed by immunoblotting with pY88-H4 antibody. The presence of a phosphorylated H4 band exclusively when ACK1 was incubated with H4 indicates the specificity of pY88-H4 antibody ([Figure 1C](#)). To further validate the H4 Y88-phospho-antibody, point mutant constructs of FLAG-tagged H4 lacking either the Tyr88 site (Y88F-H4) or the Tyr72 site (Y72F-H4) were generated. ACK1 phosphorylated H4 and the Y72F-H4, but failed to phosphorylate the Y88F-H4 mutant ([Figure 1D](#)).

To evaluate the status of endogenous H4 phosphorylation upon ACK1 activation, serum- and androgen-starved LNCaP and LAPC4 cells were treated with insulin-like growth factor (IGF) or platelet-derived growth factor. Growth factor stimulation led to robust ACK1 activation and H4 Y88-phosphorylation, both of which were suppressed by (R)-9bMS treatment ([Figure 1E](#)). To corroborate that ACK1 kinase is essential for histone H4 Y88-phosphorylation, ACK1 expression was knocked down by using small interfering RNA (siRNA) or a CRISPR-Cas9 gene editing system. ACK1 stimulation led to the phosphorylation of H4 in

control cells, and its knockdown resulted in a significant loss of H4 Y88-phosphorylation ([Figures 1F, 1G, and S3D](#)). Moreover, to explore the sensitivity of endogenous H4 Y88-phosphorylation to enzalutamide and dihydrotestosterone (DHT), serum-deprived LNCaP-C4-2B (C4-2B from hereon) and LNCaP cells were treated with vehicle, (R)-9bMS, enzalutamide, or DHT, followed by IGF treatment. The endogenous Y88-phosphorylation of H4 was eliminated upon treatment with (R)-9bMS, but was unaffected by DHT or enzalutamide treatments ([Figures 1H and 1I](#)).

ACK1 Deposits the pY88-H4 Marks Upstream of the AR Coding Region

To decipher the pY88-H4 epigenetic footprint in CRPCs, chromatin prepared from vehicle- or (R)-9bMS-treated C4-2B cells was immunoprecipitated (ChIP) with pY88-H4 antibody followed by sequencing (ChIP-seq). The pY88-H4 marks were found to be deposited at ~370 distinct locations throughout the genome ([Table S3](#)). Closer examination of X chromosome revealed pY88-H4 deposition at three distinct locations upstream of the AR transcription start site; AREM1 (nt 66, 669, 600–666, 670, 200), AREM2 (nt 66, 649, 550–566, 649, 850), and AREM3 (nt 66,

631, 300–366, 631, 650) (Figures 2A and 2B). To validate ACK1-mediated deposition of the pY88-H4 marks upstream of *AR*, ChIP was performed followed by real-time PCR using site-specific primers. The pY88-H4 marks were specifically deposited at the three AREMs but not at a control site; however, upon (R)-9bMS treatment there was a significant loss of pY88-H4 at AREMs (Figures 2C–2F), suggesting that the deposition of pY88-H4 marks is abolished by an ACK1 inhibitor.

To assess whether the pY88-H4 mark deposition is critically dependent on AR and ACK1 recruitment, C4-2B cells were transfected with AR or ACK1 siRNAs followed by ChIP-qPCR. AR was recruited at AREM1 (Figure 2G). Further, ACK1 or AR silencing significantly reduced the pY88-H4 deposition at AREM1 and AREM2 (Figures 2H and S3E). In addition, deletion of *ACK1* by CRISPR-Cas9 significantly compromised deposition of pY88-H4 marks (Figure 2I). Taken together, these data suggest that AR is needed to recruit ACK1 upstream of the *AR* locus to deposit the pY88-H4 marks.

To examine a direct role of AREMs on AR mRNA expression, AREM1 and AREM2 were deleted using a CRISPR-Cas9 gene editing method in C4-2B cells (Figure S3F). A significant reduction of AR mRNA was seen upon deletion of either AREM1 or AREM2, suggesting that these sites act as enhancers for *AR* (Figure 2J).

ACK1 Kinase Activity Is Required to Sustain AR mRNA Levels in an Androgen-Deficient Environment

Toward understanding the outcome of the androgen-independent AR/ACK1 crosstalk, we chose an unbiased approach wherein androgen-deprived VCaP, LAPC4, or C4-2B cells were treated with DHT, enzalutamide, or (R)-9bMS. Unexpectedly, we discovered that treatment with (R)-9bMS resulted in a significant downregulation of AR protein levels (Figures 3A, 3B, S3G, and S3H). In contrast, the AR antagonist enzalutamide had a modest or no effect on AR expression. To confirm that the loss of AR expression is not due to the “off target effect” of the ACK1 inhibitor, C4-2B and LNCaP cells were transfected with control and ACK1 siRNAs, or *ACK1* was deleted using the CRISPR-Cas9 gene editing technique. Loss of ACK1 expression resulted in a significant loss of AR mRNA (Figures 3C and 3D) and protein levels (Figures 3E and 3F). Further, loss of AR expression was also reflected in a decrease in prostate-specific antigen (PSA) levels (Figure 3G), suggesting that ACK1 is required for sustaining AR mRNA expression in an androgen-deficient environment.

To examine whether the deposition of pY88-H4 marks upstream of *AR* is necessary for its transcriptional upregulation, total RNA was prepared from cells that were transfected with either the Y88F mutant of H4 or WT-H4. An overexpression of the Y88F-H4 mutant resulted in a significant decrease in both AR and PSA mRNA levels (Figures 3H and 3I). Taken together, these data illustrate that the histone tyrosine kinase activity of ACK1 is required to promote AR expression in the androgen-deficient milieu of PC cells.

AR is a target of several E3 ubiquitin ligases that positively and negatively regulate its activity (Xu et al., 2009). To determine whether AR is targeted for proteasomal degradation following treatment with the ACK1 inhibitor, LAPC4 cells were treated with the proteasome inhibitor MG-132 in the presence or

absence of (R)-9bMS. Treatment with MG132 did not reverse the loss of AR expression caused by ACK1 kinase inhibition (Figure 3J), opening a possibility that ACK1 regulates AR expression at the level of transcription.

(R)-9bMS Attenuates AR and the Splice Variant-7 Transcription

To further interrogate whether the decrease in AR protein levels is a consequence of inhibition of AR transcription, PC cells grown in androgen-deprived medium were treated with vehicle, (R)-9bMS, DHT, or enzalutamide. Real-time PCR revealed that AR mRNA expression was significantly suppressed in all (R)-9bMS-treated PC cell lines (Figures 4A–4D). A modest decrease in AR mRNA levels was observed in VCaP cells following both enzalutamide and DHT treatments (Figure 4A). In contrast, AR mRNA levels were not significantly altered in DHT- or enzalutamide-treated LNCaP and LAPC4 cells (Figures 4B and 4C). Notably, as an outcome of AR transcriptional suppression by (R)-9bMS, a significant reduction in the expression of the AR target gene PSA was observed (Figures 4E–4H). As a control, LNCaP and LAPC4 cells treated with enzalutamide revealed a significant decrease in PSA expression without affecting AR expression (Figures 4B, 4C, 4F, and 4G). Intriguingly, VCaP cells displayed resistance to enzalutamide and did not exhibit reduction in PSA mRNA expression (Figure 4E). Consistent with the mechanism of action of enzalutamide to inhibit AR nuclear translocation, PSA was downregulated without affecting AR mRNA levels (Figures 4F and 4G). This is in contrast to (R)-9bMS, which reduced PSA expression by suppressing AR transcription (compare Figures 4E–4H).

It has been reported that the expression of the full-length AR is needed for the genesis of its variant, AR-V7 (Watson et al., 2010). We assessed the levels of AR-V7 mRNA and protein following treatment with (R)-9bMS. Both AR-V7 mRNA and protein levels were significantly reduced upon ACK1 inhibition (Figures 4I and 4J), suggesting that ACK1 regulates optimal AR and AR-V7 expression by a distinct epigenetic mechanism.

ACK1 Kinase Activity Drives AR Expression in an Androgen-Deficient Environment

To validate an absolute requirement of ACK1 in sustaining AR transcription, we utilized the androgen-insensitive PC3 cells, which express undetectable levels of AR and PSA. Transfection of PC3 cells with constitutively active ACK1, but not kinase-dead ACK1 (Mahajan et al., 2007), led to a detectable increase in AR protein levels (Figure 4K). This was due to a significant induction of AR (and PSA) mRNA expression (Figures 4L and 4M). Further, ChIP-qPCR analysis revealed the deposition of pY88-H4 epigenetic marks at AREM1 in ACK1-expressing cells (Figure 4N). These data indicate that ACK1 kinase activity is required to maintain AR mRNA levels in PC cells.

Attenuation of Global Androgen-Independent AR Transcriptional Program by (R)-9bMS

To determine whether AR transcriptional attenuation caused by ACK1 kinase inhibition resulted in changes in global gene expression, gene expression profiling was performed in VCaP cells treated with (R)-9bMS. Over- and underexpressed gene

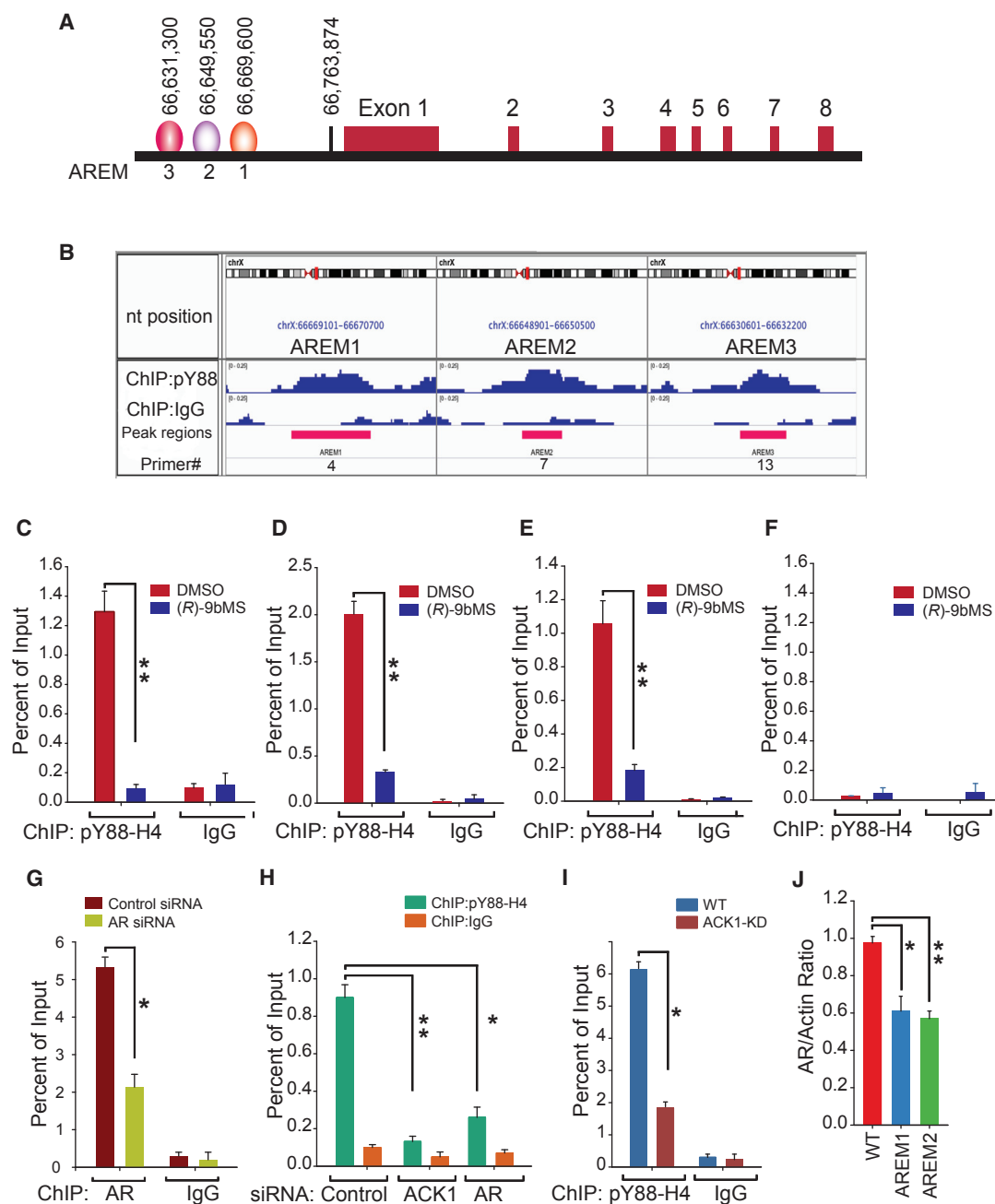


Figure 2. Deposition of pY88-H4 Epigenetic Marks Upstream of the AR Gene

(A) Chromatin was isolated from C4-2B cells, and ChIP was performed using pY88-H4 antibody or IgG followed by sequencing. The peaks upstream of the AR coding region, designated as AREM1-3 are shown in graphical format. The numbers indicate nucleotide position at the midpoint of the peaks.

(B) Peak regions that were amplified using PCR primers are shown.

(C–F) C4-2B cells were treated with vehicle or (R)-9bMS and ChIP was performed using pY88-H4 antibody or IgG, followed by qPCR using primers corresponding to AREM1(C), AREM2 (D), AREM3 (E), and control (gene desert on chromosome 12) sites (F) (n = 3, 3 replicates).

(G) C4-2B cells were transfected with control or AR siRNAs and ChIP was performed using AR antibody followed by qPCR using primers corresponding to AREM1 (n = 2, 3 replicates).

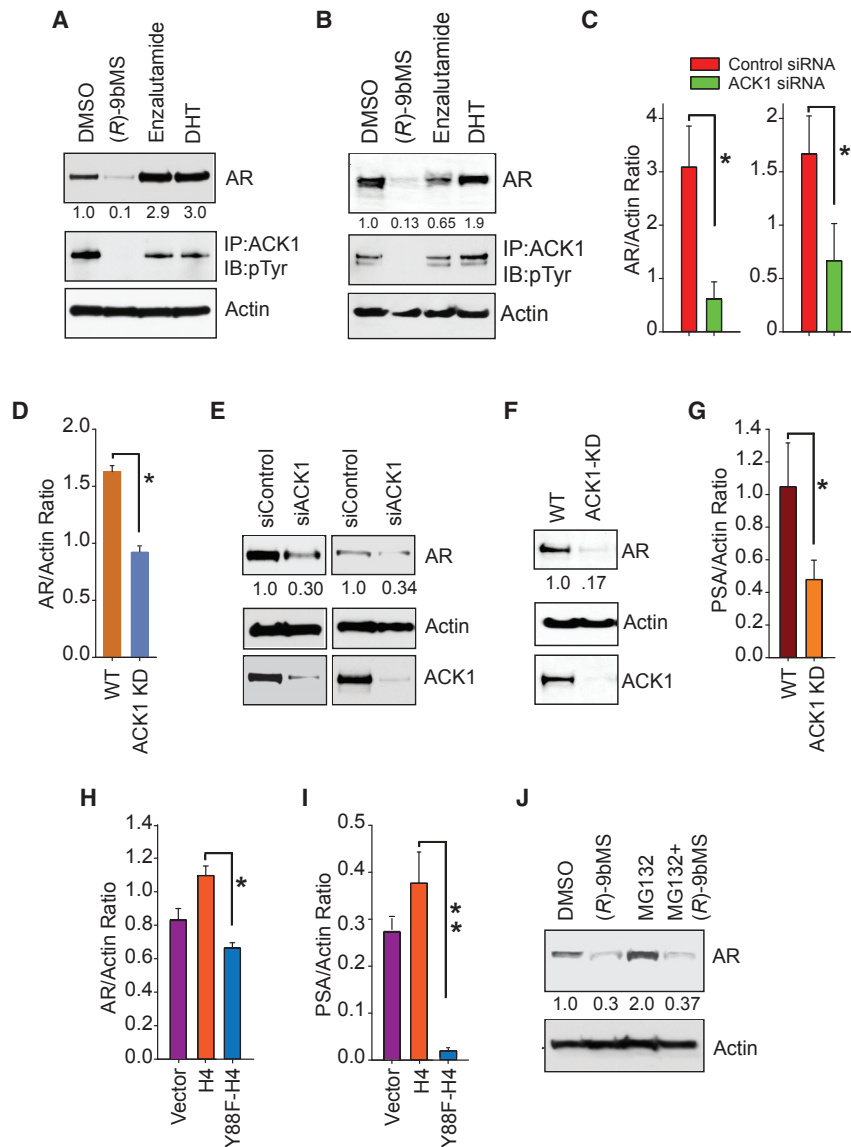
(H) C4-2B cells were transfected with control, ACK1, or AR siRNA and subjected to ChIP using pY88-H4 antibody or IgG followed by qPCR using primers corresponding to AREM2 (n = 2, 3 replicates).

(I) Chromatin isolated from ACK1 deleted C4-2B cells was subjected to ChIP with pY88-H4 antibody or IgG, followed by qPCR using primers corresponding to AREM2 (n = 2, 3 replicates).

(J) Total RNA isolated from C4-2B cells lacking AREM1 or AREM2 was subjected to qRT-PCR with AR and actin primers (n = 3, 2 replicates).

Data are represented as mean $\pm$ SEM as in (C–J). **p < 0.01, *p < 0.05.

See also Figure S3 and Table S3.



sets were generated by filtering to include data points that showed either over- or underexpression by at least 2-fold (log ratio with $p < 0.001$). A significant repression of AR expression was observed in (R)-9bMS-treated VCaP cells (Figure 5A), indicating that AR transcription is critically dependent on ACK1 kinase activity. Further, microarray followed by gene set enrichment analysis (Subramanian et al., 2005), revealed a significant decrease in DNA repair gene signature upon ACK1 inhibition (Figure 5B). In addition, inhibition of ACK1/AR signaling affected the E2F, MYC, and estrogen response (ER)-regulated transcription programs (Figures 5C–E).

To examine the differential sensitivity of PC cells to (R)-9bMS, total RNA was isolated from five PC cell lines that were either untreated or treated with (R)-9bMS, and expression profiling was performed. We curated all the AR signatures from literature and generated a heatmap. While the AR-dependent cell lines C4-2B, VCaP, and LAPC4 clustered together, the AR non-responsive cell lines PC3 and DU145 formed a separate cluster (Figure 5F),

suggesting that (R)-9bMS may exert a distinct effect on AR-expressing PCs.

To determine whether the ectopic expression of AR can rescue the androgen-independent growth inhibition by (R)-9bMS, PC cells were co-transfected with GFP and vector or FLAG-tagged AR constructs (driven by CMV promoter and thus insensitive to ACK1 inhibition), followed by (R)-9bMS treatment. Exogenous AR-expressing cells exhibited a significant survival benefit despite (R)-9bMS treatment (Figures 5G and S4A), suggesting that exogenous AR can rescue the survival phenotype due to a functional loss of ACK1.

pY88-H4-Dependent Recruitment of MLL2/WDR5 Chromatin Remodeling Complex Upstream of AR

We assessed enrichment of ChIP-seq peaks at the specific sequence motifs, which indicated that pY88-H4 deposition sites overlap with transcription factor-binding motifs and other chromatin-binding proteins (Figures S4B–S4D). To elucidate the molecular mechanism by which H4 Y88 phosphorylation regulates AR transcription, we performed an unbiased pull-down to uncover potential “readers” of the pY88-H4 marks. In brief,

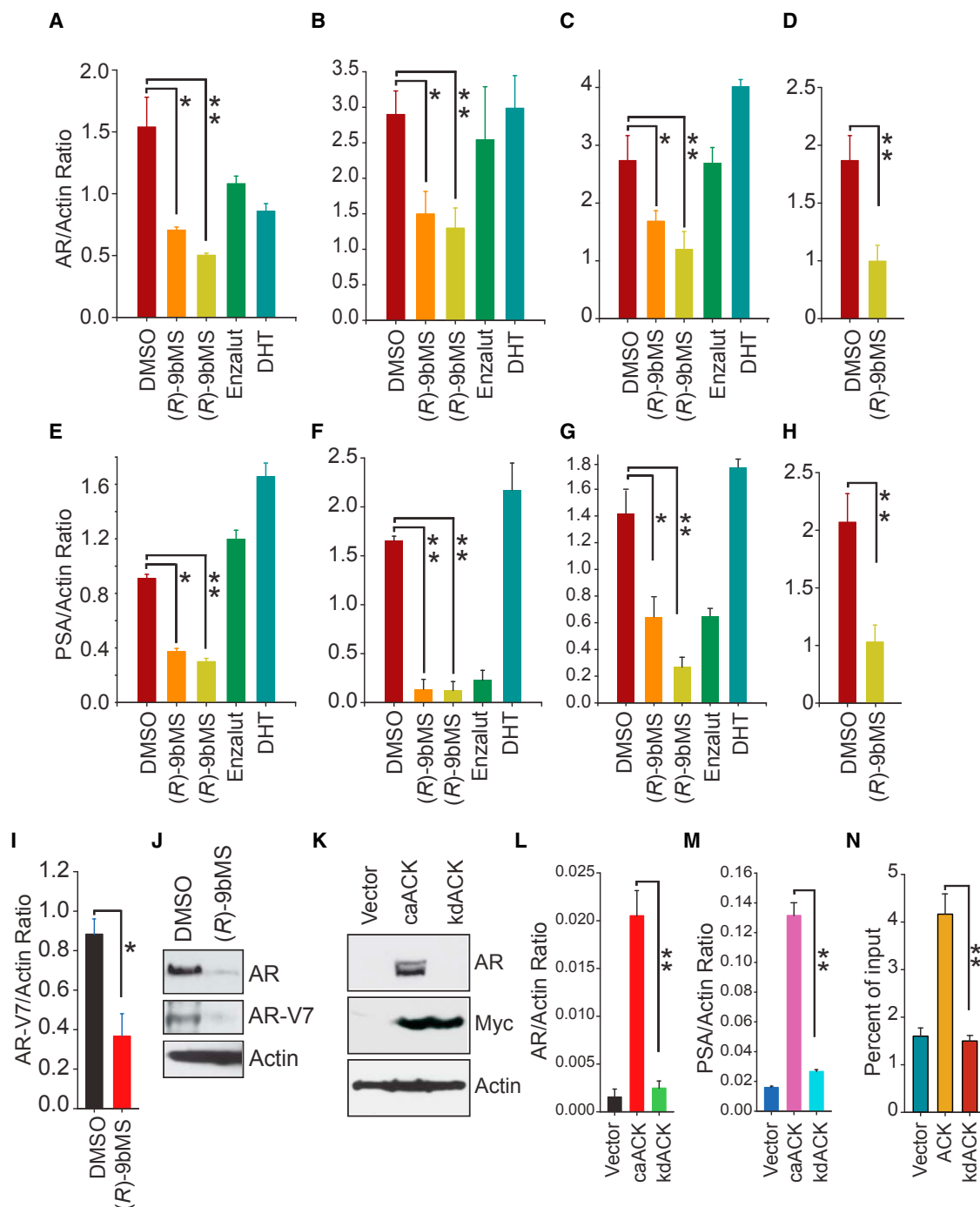


Figure 4. Inhibition of ACK1 Kinase Activity Suppresses AR Transcription

(A–D) Androgen-starved VCaP (A), LNCaP (B), LAPC4 (C), and C4-2B (D) cells were treated overnight with vehicle, (R)-9bMS at 3.5 μ M (orange) or 7 μ M (green) for 16 hr, enzalutamide (7 μ M, 16 hr), and DHT (10 nM, 2 hr). Total RNA was isolated followed by qRT-PCR with AR and actin primers (n = 3, 3 replicates).

(E–H) Total RNA isolated from androgen-starved VCaP (E), LNCaP (F), LAPC4 (G), and C4-2B (H) cells treated as described above and subjected to qRT-PCR with PSA and actin primers (n = 3, 3 replicates).

(I) Total RNA isolated from vehicle- or (R)-9bMS-treated VCaP cells followed by qRT-PCR with AR-V7 and actin primers (n = 2, 3 replicates).

(J) The lysate prepared from vehicle- or (R)-9bMS-treated VCaP cells were subjected to immunoblotting with indicated antibodies.

(K) PC3 cells were transfected with MYC-tagged constitutively active ACK1 (caACK), kinase-dead ACK1 (kdACK), or vector, followed by immunoblotting.

(L and M) Total RNA was isolated from PC3 cells transfected as described in (K), followed by qRT-PCR with AR (L) and PSA (M) and actin primers (n = 2, 3 replicates).

(N) Chromatin isolated from PC3 cells transfected with full-length ACK, kdACK, or vector cells were subjected to ChIP with pY88-H4 antibody, followed by qPCR using primers corresponding to AREM1 (n = 2, 3 replicates).

Data are represented as mean $\pm$ SEM as in (A–I, L–N). **p < 0.01, *p < 0.05.

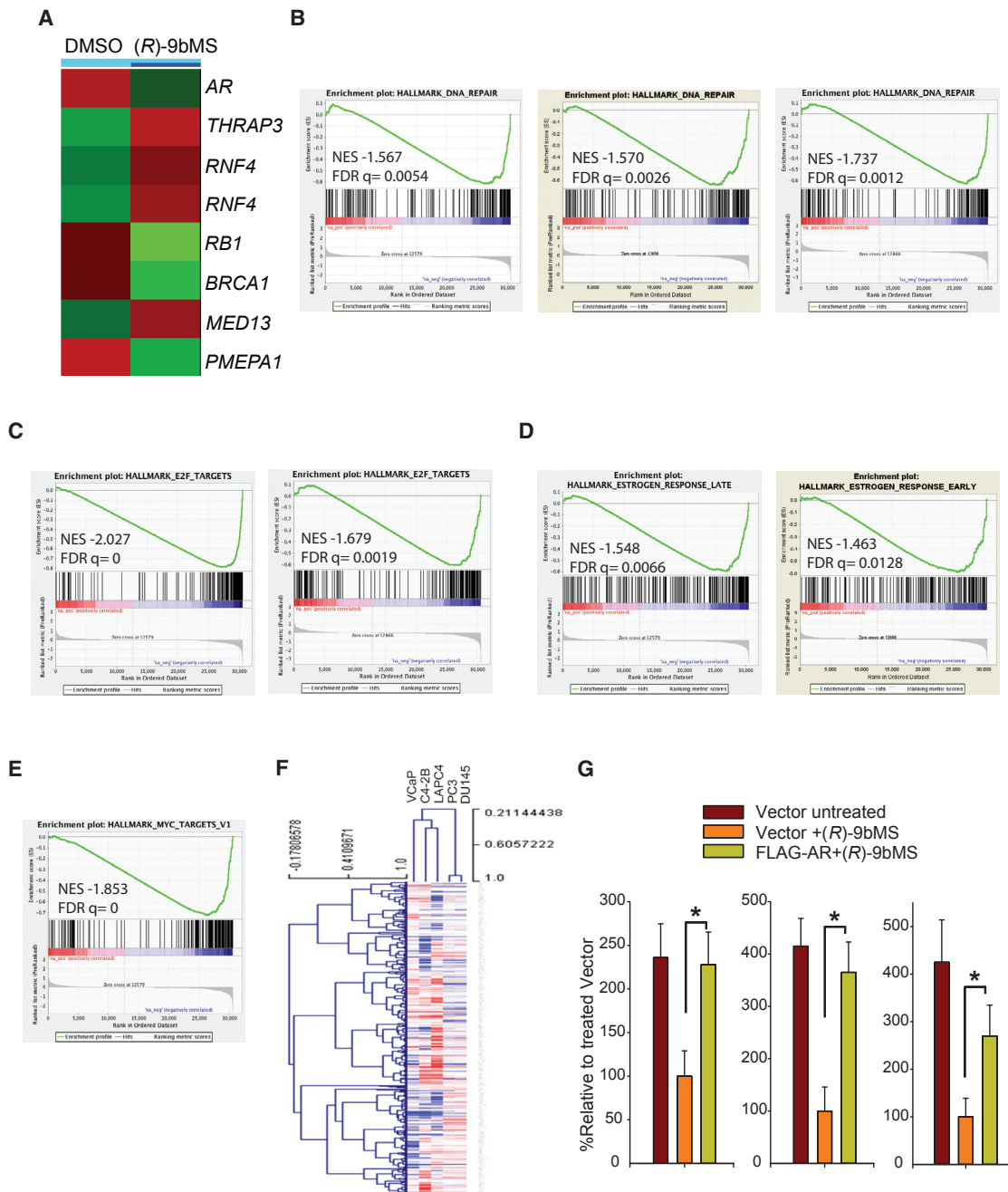


Figure 5. ACK1 Regulates AR Expression and DNA Repair, and E2F and MYC Target Gene Signatures

(A) Heatmap of microarray expression data of VCaP cells treated with vehicle or (R)-9bMS.

(B) Gene set enrichment analysis (GSEA) of the DNA repair target gene signature in C4-2B (left), VCaP (middle), and LAPC4 (right) cells impacted by (R)-9bMS treatment.

(C) GSEA of the E2F target gene signature in C4-2B (left) and LAPC4 (right) cells impacted by (R)-9bMS treatment.

(D) GSEA of the estrogen response gene signature in C4-2B (left) and VCaP (right) cells impacted by (R)-9bMS treatment.

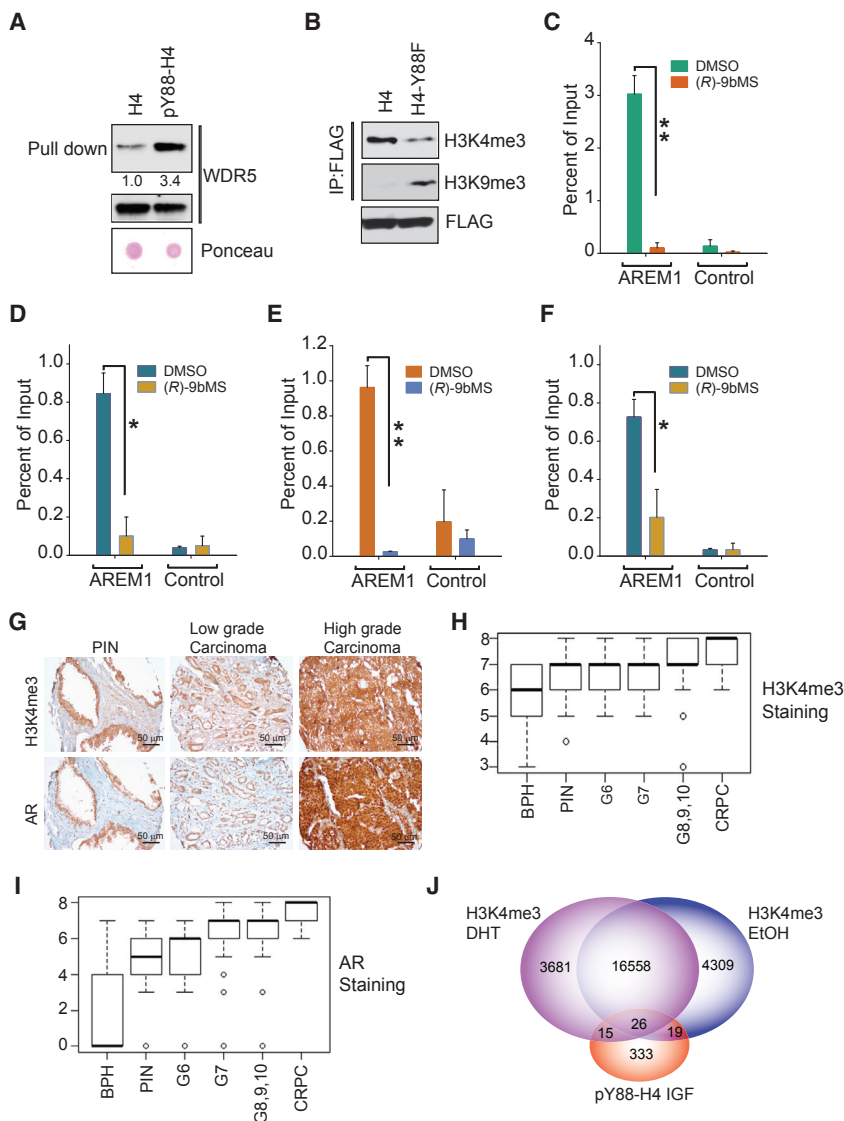
(E) GSEA of the MYC target gene signature in C4-2B cells impacted by (R)-9bMS treatment.

(F) Heatmap of all the curated AR signatures in C4-2B, VCaP, LAPC4, PC3, and DU145 cells.

(G) Androgen-deprived LAPC4 (left), VCaP (middle), and LNCaP (right) cells were co-transfected with GFP and either FLAG-tagged AR or vector constructs, followed by treatment with vehicle or (R)-9bMS (3 μ M, 96 hr). GFP-positive cells were counted after 96 hr.

Data are represented as mean $\pm$ SEM (n = 3, 3 replicates). *p < 0.05.

See also Figure S4.



biotinylated histone H4-derived peptide with a phosphorylated Y88 residue at the center (amino acids 80–96) and an equivalent unphosphorylated peptide (as control) were immobilized onto streptavidin beads, and bound proteins were analyzed by liquid chromatography-tandem mass spectrometry analysis. The top “hit” in this proteomic analysis was MLL2 (KMT2D), a H3K4-methyltransferase (Table S4). In addition MLL2-interacting proteins WDR5 and ASH1L were also present in the pull-down (Table S4; Figures S5A and S5B).

The WD40 repeat protein WDR5 is a key component of the MLL2 H3K4-methyltransferase chromatin remodeling complex, and is required for binding of the methyltransferase complex to the K4-dimethylated H3 tail-facilitating global H3K4 trimethylation (Wysocka et al., 2005). The H3K4 trimethylation is associated with transcriptional activation in a number of contexts, and the mono-, di-, and trimethylated forms of H3K4 are known to be differentially enriched at the promoter, enhancer, and other regulatory sequences (Wysocka et al., 2005; Zhao et al., 2007). Thus, our proteomics analysis provided a key clue; the H4 Y88

for WDR5. WDR5 exhibited preferential binding to the phosphorylated Y88-H4, compared with the unphosphorylated H4 (Figure 6A).

To further validate a direct role of pY88-H4 in the recruitment of the WDR5/MLL2 complex, chromatin prepared from either the FLAG-tagged H4 or the Y88F-H4 mutant transfected cells was immunoprecipitated with FLAG beads, followed by analysis of the H3K4 trimethylation status. Chromatin extracted from the mutant Y88F-H4-expressing cells displayed a significant decrease in the H3K4me3 marks compared with the WT-H4-expressing cells (Figure 6B). Moreover, H3K9 trimethylation, known to be transcriptionally repressive, was significantly increased in the H4 Y88F mutant-expressing cells (Figure 6B), suggesting that the interplay of activating/repressive methylation may fine-tune AR mRNA output in PC cells.

To determine the physiological relevance of the pY88-H4 interaction with the WDR5/MLL2 complex, we analyzed the localization of WDR5, MLL2, H3K4me1, H3K4me2, H3K4me3, and RNA Pol II at the AREM1-3 sites. ChIP-qPCR analysis

phosphorylation marks may be recognized by WDR5 and could operate in *trans* by facilitating the recruitment of MLL2, followed by H3K4 trimethylation. To verify the relevance of these epigenetic interactions, we performed a pY88-H4 peptide pull-down assay, followed by immunoblotting

revealed a specific recruitment of WDR5, MLL2, and RNA Pol II, and enrichment of H3K4me3 at the AREM1-3, which was abolished following treatment with (*R*)-9bMS (Figures 6C–6F and S5C–S5G). In contrast, the deposition of H3K4me1 and H3K4me2 was not impacted by the loss of pY88-H4 (Figure S5H). Collectively, these data indicate that activated ACK1 establishes a transcriptionally permissive chromatin landscape rich in H4-Y88 and H3K4me3 to promote AR mRNA transcription in the androgen-deficient environment of CRPCs.

H3K4 Trimethylation and AR Expression Correlate with Disease Progression in Metastatic Prostate Cancer

To determine the clinical relevance of H3K4me3 mark deposition on AR protein expression, we performed immunohistochemical staining of clinically annotated prostate ($n = 250$) tissue microarrays with respective antibodies. The H3K4me3 and AR levels increased as PCs progressed from the benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PINs), G6, G7, or G8-10 PCs to the CRPC stage (Figures 6G–6I). In addition, H3K4me3 and AR expression were found to positively correlate with each other during disease progression (Pearson correlation $r = 0.66$, $p < 0.0001$). Moreover, the expression of H3K4me3 and AR differed significantly between the stages of the disease; a significant increasing trend of intensity across progression stages was detected (Mantel-Haenszel test, $p = 0.01$) (Table S5 and Table S6). Based on the pairwise comparisons using Tukey's multiple adjustment method G7, G8/9/10, and CRPC groups showed a higher mean value of H3K4me3 level compared with the BPH group.

The expression of H3K4me3 in the CRPC set was also significantly higher than PIN, G6, and G7 groups. Moreover, the mean AR expression in G7, G8/9/10, and CRPC groups were all significantly higher than BPH, PIN, and G6 groups (Table S5 and Table S6). We also assessed whether the peaks corresponding to the pY88-H4 epigenetic marks overlap with the previously reported H3K4me3 deposition in DHT- or EtOH-treated C4-2B cells and observed that 60 pY88-H4 peaks overlapped with H3K4me3 peaks (Figure 6J). In addition, pY88-H4 levels positively correlated with AR expression during disease progression (Pearson correlation $r = 0.3$, $p < 0.0001$) (Table S7). Collectively, these data indicate that the expression of pY88-H4, H3K4me3, and AR progressively increase as disease progressed to later stages, with the highest expression in CRPCs. Further, it indicates that H3K4me3 marks may have a direct impact on AR expression in metastatic PC.

Targeting the ACK1 Epigenetic Activity Mitigates CRPC Tumor Growth

To investigate the anti-tumor activity of ACK1 inhibitor, we first analyzed the ability of (*R*)-9bMS to suppress the proliferation of PC cell lines. AR-positive cell lines C4-2B, VCaP, LAPC4, and LNCaP were found to be sensitive to the (*R*)-9bMS treatment, with IC_{50} values of 400, 450, and 750 nM, and 1.8 μ M, respectively (Figure 7A). Interestingly, CRPC-forming C4-2B and VCaP were the most sensitive, while the “normal” prostate cells, RWPE, were the most resistant to (*R*)-9bMS treatment ($IC_{50} \sim 10 \mu$ M). To examine the consequences of (*R*)-9bMS-mediated AR mRNA transcription suppression, cell-cycle analysis was performed. A significant increase in the number of cells in

G₂/M phase was seen following (*R*)-9bMS treatment (Figure 7B). To assess whether this G₂/M arrest could lead to the activation of apoptotic pathways, tyrosine kinase inhibitor-treated cells were stained with caspase-3/7 detection agent and SYTOX dead cell stain. In contrast to the selective SRC inhibitor, saracatinib (22%), a significant increase in apoptotic cells (52%) was observed following treatment with (*R*)-9bMS (Figure S6A).

Among the various PC cell lines, C4-2B cells display high sensitivity to enzalutamide (Figure S6B). To examine the relative sensitivity of CRPCs to enzalutamide and (*R*)-9bMS, we generated an enzalutamide-resistant C4-2B cell line that grows in the presence of 25 μ M of enzalutamide. Unexpectedly, enzalutamide at lower concentrations acted as an agonist and fueled the proliferation of enzalutamide-resistant C4-2B cells (Figure 7C). Interestingly, a significant increase in AR mRNA expression was observed in enzalutamide-resistant C4-2B cells, suggesting that CRPCs could overcome enzalutamide blockade by reinvigorating AR expression (Figure 7D). Although these cells exhibited considerable resistance to enzalutamide ($IC_{50} > 7.5 \mu$ M), cells retained sensitivity to (*R*)-9bMS (Figure 7C) by virtue of its ability to suppress AR expression (Figure 7E). Taken together, these data suggest that ACK1/AR signaling can overcome enzalutamide resistance by downregulating AR transcription.

To investigate the effect of (*R*)-9bMS on CRPC xenograft tumor growth, C4-2B cells were implanted subcutaneously in castrated male SCID mice. When the tumors reached approximately 100 mm³ in size, the mice were randomized and injected with either the vehicle or (*R*)-9bMS. Although vehicle-treated mice formed robust subcutaneous CRPC tumors, tumor growth was not observed in the (*R*)-9bMS-injected mice (Figures 7F, 7G, and S6C). Interestingly, metastatic tumors were observed at distant sites in some mice; two large tumors were seen attached to the prostate in the vehicle-treated mice (Figure S6C). In contrast, a small tumor was found to be attached to the inner side of the rib cage in (*R*)-9bMS-injected mice (Figure S6C), suggesting that (*R*)-9bMS may also inhibit metastatic CRPC tumor growth. Further, total RNA was isolated from the xenograft tumors from the vehicle- and (*R*)-9bMS-treated group (three small tumors were pooled from (*R*)-9bMS-injected mice from one of the two repeats) to examine AR and the expression of its target genes. The CRPC tumors from (*R*)-9bMS-treated mice exhibited a significant reduction in AR, PSA, and TMPRSS2 mRNA levels compared with the vehicle-treated group (Figure 7H).

We also assessed the effect of (*R*)-9bMS in another CRPC xenograft model, LNCaP-caAck (Mahajan et al., 2007). Similar to C4-2B, LNCaP-caAck CRPC tumors responded to (*R*)-9bMS treatment with a significant reduction in xenograft tumor growth (Figure S6D).

Markedly, the (*R*)-9bMS treatment regimen was well tolerated by mice; a small but insignificant weight loss was noticed in (*R*)-9bMS-treated mice compared with the vehicle-treated mice (Figure S6E). To further assess any pathological effect of (*R*)-9bMS treatment, heart, spleen, kidneys, and livers from the mice were excised and stained with H&E. The organs from vehicle- and (*R*)-9bMS-treated mice did not reveal histological abnormalities (Figure 7I).

As an initial response to ADT, the prostate tumors shrink with a significant decrease in PSA levels (Grossmann et al., 2001).

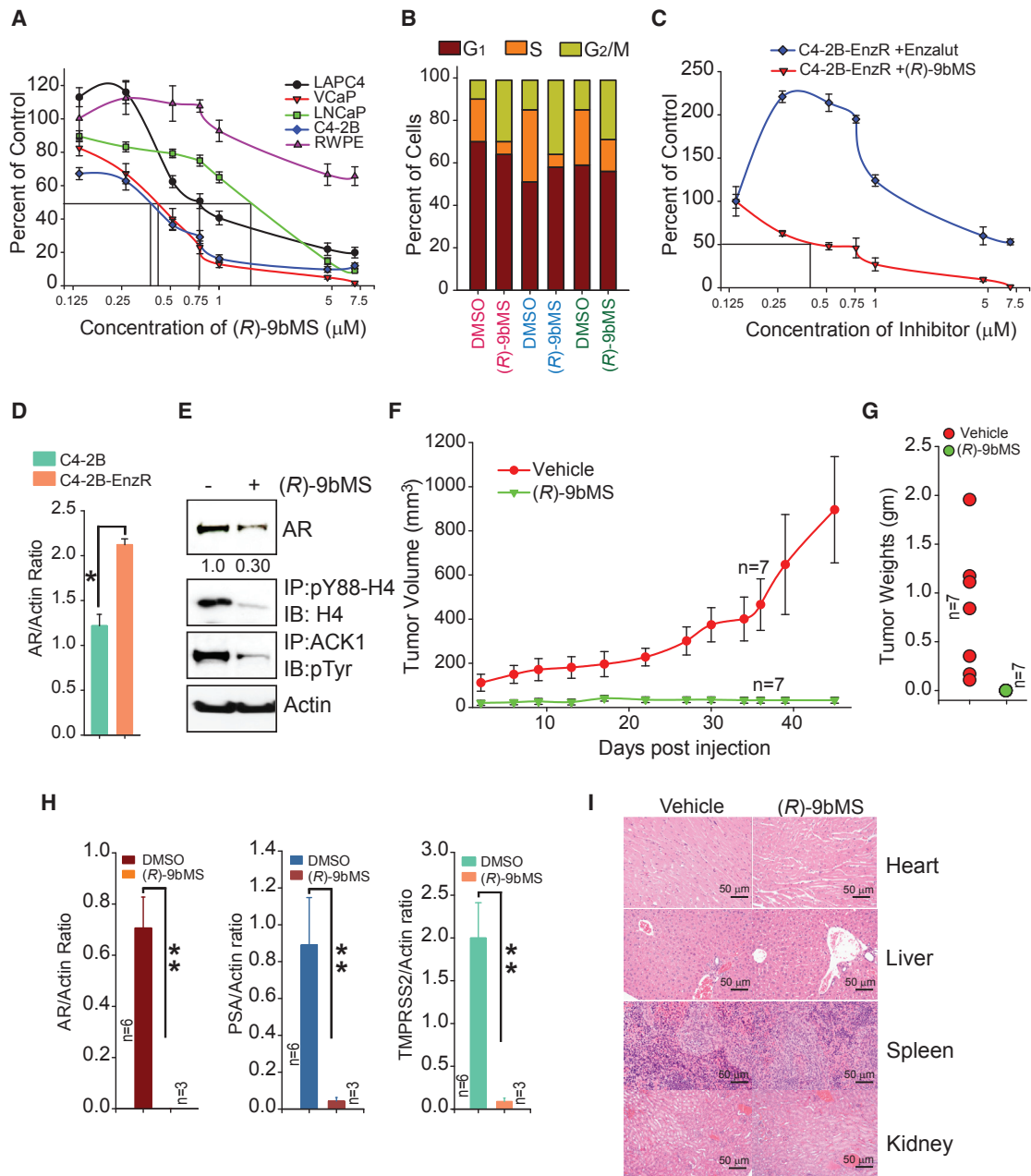


Figure 7. (R)-9bMS Overcomes Enzalutamide Resistance and Inhibits CRPC Tumor Growth

(A) Cells were treated with (R)-9bMS or vehicle (10% DMSO) for 96 hr and the number of viable cells counted by trypan blue exclusion assay ($n = 2, 3$ replicates). (B) Flow cytometry-based cell-cycle analysis of LNCaP (labeled in red), LAPC4 (blue), and C4-2B (green) cells treated with vehicle or (R)-9bMS for 24 hr and stained with propidium iodide.

(C) Enzalutamide-resistant C4-2B cells were treated with enzalutamide or (R)-9bMS for 96 hr and the number of viable cells was determined by trypan blue dye exclusion assay ($n = 2, 3$ replicates).

(D) Total RNA isolated from parental and enzalutamide-resistant C4-2B cells was subjected to qRT-PCR with AR and actin primers ($n = 2, 3$ replicates).

(E) Enzalutamide-resistant C4-2B cells were treated with vehicle (–) or (R)-9bMS (+), and immunoblotting was performed with indicated antibodies.

(F) C4-2B cells were implanted subcutaneously in castrated male SCID mice. When tumors became palpable, mice were injected either with vehicle (10% DMSO in PBS) or (R)-9bMS (50 mg/kg of body weight) for 5 days a week for 4 weeks ($n = 7$ mice for each treatment). Tumor volumes were measured with calipers.

(G) The weights of the xenograft tumors in (F) are shown.

(H) Total RNA was prepared from xenograft tumors followed by qRT-PCR with AR (left), PSA (middle), and TMPRSS2 (right) primers ($n = 2, 3$ replicates).

(I) Heart, liver, spleen, and kidneys were harvested from vehicle- or (R)-9bMS-treated mice, fixed and sectioned followed by H&E staining.

Data are represented as mean $\pm$ SEM as in (A, C, D, F–H). ** $p < 0.01$, * $p < 0.05$.

See also [Figures S6](#) and [S7](#)

Paradoxically, CRPC tumors “learn” to thrive under castrate levels of androgen. A pervasive mechanism in CRPCs appears to be to overexpress AR, suggesting that CRPCs have eruditely dealt with dwindling androgen levels. But, how does AR sustain its own expression in the absence of ligand? Interestingly, androgen deprivation of androgen-sensitive LAPC4 cells for 10 days resulted in a significant upregulation of ACK1 levels (Figure S6F). Activation of ACK1 leading to H4 Y88-phosphorylation, resulting in AR transcriptional activation, seems to be interlinked in the androgen-deficient environment (Figures S6G and S6H). Importantly, expression of the Y88F-H4 mutant in LNCaP cells significantly compromised growth in androgen-deficient medium (Figure S7A). Moreover, castrated mice injected with the C4-2B cells expressing a Y88F mutant of H4 exhibited a reduction in xenograft tumor growth and PSA levels compared with H4- or vector-expressing cells (Figures S7B–S7D). Although, cells seem to be equally adept in incorporating Y88F-H4 as well as H4 in chromatin, consistent with its reader function, MLL2/WDR5 recruitment was significantly compromised at AREM when mutant Y88F-H4 was overexpressed (Figures S7E–S7G). A frequent phenomenon in CRPC is that AR and ACK1 gene amplification is seen in ~70% and 22% of these tumors, respectively (Figures S7H–S7J); indeed, AR and ACK1 exhibit significant tendency toward co-occurrence in CRPCs ($p = 0.002$, log odds ratio 1.68) (Figure S7K). Taken together with data shown in Figure S6F, these results indicate that CRPCs adapt to androgen-deficiency by selectively favoring ACK1/AR expression.

DISCUSSION

Here we demonstrate chromatin-directed transcriptome reprogramming of the cancer cells as a paradigm for the emergence of drug-resistant and lethal forms of PC. We uncovered that ACK1 directly reprograms the AR locus to promote castration and drug resistance. Further, we have demonstrated that ACK1-AR signaling sustains expression of genes important for DNA repair cell cycle by regulating E2F target genes, and cell proliferation by MYC regulation, to promote malignancy. This is consistent with earlier reports demonstrating a role for AR in the regulation of DNA repair genes in PCs (Goodwin et al., 2013; Mahajan et al., 2012a; Polkinghorn et al., 2013; Spratt et al., 2015). AR has been shown to regulate the MYC transcription program in an androgen-independent manner (Gao et al., 2013), which explains the significant loss of MYC target gene expression upon (R)-9bMS treatment. Intriguingly, these data also suggest a role for ACK1 in driving ER-mediated transcription, which is consistent with our earlier observation wherein ACK1 interacts with ER and its co-activator histone demethylase KDM3A (Mahajan et al., 2014). Combined, these results illustrate that targeting of major survival pathways by the ACK1 inhibitor underlie its effectiveness in restraining the growth of PC cells.

PC is considered as a cancer of the epigenome (Grasso et al., 2012; Robinson et al., 2015). Our results demonstrate that the interaction of AR with ACK1 drives the positive feedback epigenetic circuitry that is ultimately conducive to promote AR transcription. Further, this circuitry subjugates AR transcription in CRPCs when the H4-Y88 epigenetic regulation is terminated. An unexpected finding that has emerged from this study is the

role of ACK1 as an “epigenetic writer” that deposits the first layer of pY88-H4 marks, which are recognized by an “epigenetic reader” WDR5. This, in turn, results in the recruitment of the WDR5 partner MLL2, which acts as the “epigenetic scribe” and introduces H3K4me3-activating marks to drive AR expression. Interestingly, recent studies have shown that AR interacts with menin, which is a component of the MLL1/WDR5/ASH2L complex (Grasso et al., 2012; Malik et al., 2015). Whether H4 Y88phosphorylation was important for the assembly of MLL1/WDR5/menin complex is not known; however, the presence of WDR5 in both MLL1 and MLL2 complexes does not rule out the possibility of the global regulatory function of pY88-H4 marks in regulation of the AR target genes. Significantly, our proteomics analysis revealed many other WD repeat-containing proteins, including WDR55, WDR43, WDR19, WDR12, WDR46, and WDR75, also binding to pY88-H4, opening the possibility that WD40 repeat could be the pY88-H4 reader element and that WD40 repeat-containing proteins might be recruited to chromatin using these epigenetic marks.

In addition to WDR5, our assessment of pY88-H4 ChIP-seq peaks revealed the top match being the SOX8 (or sex determining region Y-box 8), a transcription factor involved in the regulation of embryonic development and determination of the cell fate, including male sex determination (Chaboissier et al., 2004). Whether pY88-H4 marks have an additional role in male gonad differentiation remains to be seen.

Prompt restoration of AR expression reinforces the notion that sustained androgen deprivation may prompt CRPC tumors to use ACK1/pY88-H4 epigenetic signaling to overcome androgen dependence. Although ACK1/pY88-H4/AR signaling could be the promising scheme for CRPCs to acquire the above-mentioned properties; the unavailability of an ACK1 small-molecule inhibitor precluded testing this hypothesis comprehensively. Our data not only provide evidence of CRPC dependence on ACK1-AR signaling, but also indicates that enzalutamide-non-responsive PCs may be sensitive to the ACK1 inhibitor. Indeed, combining enzalutamide with (R)-9bMS might provide the best outcome, dampening the progression of early disease to castration resistance. Overall, uncovering the epigenetic control of CRPCs has considerable potential to advance (R)-9bMS as a therapeutic option.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccell.2017.05.003>.

AUTHOR CONTRIBUTIONS

Conceptualization, K.M. and N.P.M.; Methodology, K.M., H.R.L., N.J.L., and N.P.M.; Investigation, K.M., P.M., and N.P.M.; Formal Analysis, Z.C., J.K., and D.C.; Resources, K.M., H.R.L., N.J.L., and N.P.M.; Data Curation, C.K.S., R.M., and S.S.; Original Draft, K.M. and N.P.M.; Review & Editing, K.M. and N.P.M.; Supervision, K.M. and N.P.M.; Project Administration, N.P.M.; Funding Acquisition, K.M. and N.P.M.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
AR	Biogenex	Cat #AM256
AR(D6F11)	Cell signaling	Cat#5153; RRID: AB_10691711
AR ChIP grade	Abcam	Cat#ab74272; RRID: AB_1280747
AR-V7 antibodies	Precision	Cat #AG10008; RRID: AB_2631057
WDR5	Active Motif	Cat #61485
H3K4me1 (D1A9)	Cell Signaling	Cat #5326; RRID: AB_10695148
H3K4me2 (C64G9)	Cell Signaling	Cat #9275; RRID: AB_10205451
H3K4me3	Active motif	Cat#39915
MLL2	Santacruz	Cat#SC-293217
ACK1	Santacruz	Cat#SC-28336; RRID: AB_626629
Phospho-tyrosine-HRP	Santacruz	Cat#SC-508; RRID: AB_628122
pACK1 (Tyr284)	Upstate	Cat#09-142; RRID: AB_612088
Actin monoclonal antibody	Sigma-Aldrich	Cat#A5441; RRID: AB_476744
H4 polyclonal antibody	Genscript	Cat#A01176-40; RRID: AB_1237588
RNA Pol II	Active Motif	Cat#61667
IgG antibodies	Abcam	Cat#ab2410; RRID: AB_303052
Histone H2A monoclonal antibodies (L88A6)	Cell Signaling	Cat#3636; RRID: AB_2118801
H2B monoclonal antibodies	Cell Signaling	Cat#2934; RRID: AB_2295301
H3 monoclonal antibodies	Cell Signaling	Cat#3638; RRID: AB_10828254
H4 monoclonal antibodies	Cell Signaling	Cat#2935; RRID: AB_1147658
Anti-Mouse secondary antibodies	Promega	Cat#W402B
Goat-anti-rabbit IgG	Epitomics	Cat#3053-1; RRID: AB_1580740
Alexa Fluor 488 conjugated anti-rabbit secondary antibody	Invitrogen	Cat#R37116; RRID: AB_2556544
Alexa Fluor 647 conjugated anti-mouse secondary antibody	Invitrogen	Cat#A32728; RRID: AB_2633277
SYBR Green RT-PCT Reagent kit	Applied Biosystem	Cat#4310179
SYBR PremixEx Taq II (Tli RNase H Plus)ROX	Clontech Takara	Cat#RR82LR
Anti-HA mouse monoclonal	Cell signaling	Cat#2367; RRID: AB_10691311
Anti-FLAG mouse monoclonal antibody	Sigma	Cat#F3165; RRID: AB_259529
Bacterial and Virus Strains		
DH5a subcloning efficiency	Invitrogen	Cat#18265017
Biological Samples		
IGF	Peptrotech	Cat#100-11
PDGF	Invitrogen	Cat#PHG0035
rhEGF	Invitrogen	Cat# PHG0311
Insulin	Sigma	Cat#10516-5ml
Fetal Bovine Serum	Life technologies	Cat#160044
Recombinant ACK1 protein	Lawrence et al., 2015	N/A
Matrigel	Corning/Fisher	Cat#354428
Chemicals, Peptides, and Recombinant Proteins		
(R)-9bMS	Moffitt Cancer Center	N/A
Enzalutamide	Selleck Chemicals	Cat#S1250
DHT (Dihydrotestosterone)	Sigma	Cat#A8380
Trypan Blue	Sigma	Cat #T8154

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DMSO (Dimethyl sulphoxide)	Sigma	Cat#D2650
H4 peptide	Moffitt Proteomics Core	N/A
pY88-H4 peptide	Moffitt Proteomics Core	N/A
Human H2A recombinant protein	New England Biolabs	Cat#M2502S
Human H2B recombinant protein	New England Biolabs	Cat#M2505S
Human H3 recombinant protein	New England Biolabs	Cat#M2506S
Human H4 recombinant protein	New England Biolabs	Cat#M2504S
EDTA	Sigma	Cat#E7889
DTT	Sigma	Cat#43815
ATP	Sigma	Cat#A6559
HEPES	Sigma	Cat#H3537
Glycerol	Sigma	Cat#G9012
Streptavidin-sepharose beads	Cell Signaling	Cat#3419
Triton X-100	Sigma	Cat#T9284
Glycine	Sigma	Cat#G7126
Sodium chloride	Sigma	Cat#71386
Protease Inhibitor-EDTA free	Roche	Cat#11836170001
Protein-G magnetic beads	Bio-Rad	Cat#161-4023
Protein- A magnetic beads	Bio-Rad	Cat#161-4013
Formaldehyde	Sigma	Cat#F8775
SYBR Green PCR Master Mix	Applied Biosystems	Cat#4309155
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Labs	Cat#H-1200
Insulin (from bovine pancreas)	Sigma	Cat#I5500
T3 (3'3', 5-triiodo-L-thyronine)	Sigma	Cat#T2752
apo-Transferrin	Sigma	Cat#T4382
Protein A/G agarose beads	Santacruz	Cat# sc-2003
MG132 Proteasome inhibitor	Selleck Chemicals	Cat#S2619
B-Mercaptoethanol	Sigma	Cat#M3148
Sodium OrthoVanadate	Fisher	Cat#S454-50
Sodium Fluoride	Sigma	Cat#S6521
Critical Commercial Assays		
Histone Purification Kit	Active motif	Cat#40025
MODified Histone Peptide Array	Active motif	Cat#13001
ChIP-IT Express	Active motif	Cat#53008
RNeasy plus kit	Qiagen	Cat#74134
MinElute PCR purification kit	Qiagen	Cat#28004
HiSpeed Plasmid Maxi Kit	Qiagen	Cat#12663
Miniprep Kit	Qiagen	Cat#27106
Pierce ECL Western Blotting Kit	ThermoFisher	Cat#32106
Pierce ECL Plus Western Blotting Kit	ThermoFisher	Cat#32132
Deposited Data		
GSE79658	This paper	GEO database
GSE79689	This paper	GEO database
Experimental Models: Cell Lines		
LNCaP	ATCC	ATCC® CRL-1740
LAPC4	Bob Reiter/UCLA	N/A
VCaP	ATCC	ATCC® CRL-2876
PC3	ATCC	ATCC® CRL-1435

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
C4-2B	Dr. Evan Keller, University of Michigan	N/A
DU145	ATCC	ATCC® HTB-81
RWPE	ATCC	ATCC® CRL-11609
Experimental Models: Organisms/Strains		
Mice-SCID	Charles River	Strain code:236
Athymic nude mice	Charles River	Nu/Nu Strain code:088
Oligonucleotides		
AR CHIP FP4: TAGGAATCCAACTTACAAAG GAAGTGAAGG	This paper	N/A
AR CHIP RP4: GCAGTATGGCCATTTTCACG ATATCAATTA	This paper	N/A
AR CHIP FP7: ATATCATCCCATGGCCTCTG GCTTCCATCA	This paper	N/A
AR CHIP RP7: AGTGACACTGAGACAAATTAT AAGAAAACT	This paper	N/A
AR CHIP FP13c:GGGATTCGTTGTGCAGATT ATTTTCATCACCTAGG	This paper	N/A
AR CHIP RP13c:GCCAAATGATGAGAACACA TGGACACATATAAGGG	This paper	N/A
Control ChIP FP:	Active Motif	Cat#71001
Control ChIP RP:	Active Motif	Cat#71001
qRT-PCR Primers:	This paper	N/A
AR FP: ATGGTGAGCAGAGTGCCCTATC	This paper	N/A
AR RP: ATGGTCCCTGGCAGTCTCCAAA	This paper	N/A
AR-V7 FP: CAGGGATGACTCTGGGAGAA	This paper	N/A
AR-V7 RP: GCCCTCTAGAGCCCTCATT	This paper	N/A
ACK1 FP: ACTTTGGGCTGATGCGAGCACT	This paper	N/A
ACK1 RP: AAGGTGCGTGTCTTCAGGCTCT	This paper	N/A
SRC FP: CTGCTTTGGCGAGGTGTGGATG	This paper	N/A
SRC RP: CCACAGCATACAACCTGCACCAG	This paper	N/A
ACTIN FP: CACCATTGGCAATGAGCGGTTC	This paper	N/A
ACTIN RP: AGGTCTTTGCGGATGTCCACGT	This paper	N/A
PSA FP: CGCAAGTTCACCTCAGAAGGT	This paper	N/A
PSA RP: GACGTGATACCTTGAGCACACC	This paper	N/A
TMPRSS2 FP: CAGGAGTGACGGGAATGTG ATGGT	This paper	N/A
TMPRSS2 RP: GATTAGCCGTCTGCCCTCATT TGT	This paper	N/A
gRNA Primers:		
AR1_0_219_TGG: ATAATTGATATCGTGA AAATGG (For AREM1 site)	This paper	N/A
UP2_0_107_CCC: CCCATGGCCTCTGGCT TCCATC (For AREM2 site, left)	This paper	N/A
UP2_0_201_TGG: TTCGGGTGGTCTCTGC CTTTGG (for AREM2 site, right)	This paper	N/A
Recombinant DNA		
MYC & FLAG tagged Histone H4	Origene	Cat#RC210850
FLAG-tagged AR	Mahajan et al., 2007	N/A
Myc-tagged caAck	Mahajan et al., 2005	N/A
Myc-tagged kdAck	Mahajan et al., 2005	N/A
HA-tagged ACK1	Mahajan et al., 2007	N/A
Software and Algorithms		
Sigma Statistical Software 13.0	SigmaPlot	N/A
SDS software version 2.2.2	Applied Biosystems	
JAVA program/ Gene Set Enrichment Analysis		Molecular Signatures Database (MSigDB)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agilent Whole Human Genome Oligo Microarray	Agilent Genomics	Cat#G4112A
Flow Jo Software	FLOWJO LLC	FlowJo
ABI PRISM 7900HT Sequence Detection System	Applied Biosystems	N/A
Axiovision version 4.8 software	Carl Zeiss	N/A
Other		
DMEM low glucose	Gibco	Cat #11885-084
F12	Gibco	Cat#11765-054
RPMI	Gibco	Cat#11875-119
RPMI (No phenol)	Gibco	Cat#11835-0355
DMEM	Gibco	Cat#11965-092
IMDM	Gibco	Cat#12440-053
Trypsin-EDTA (0.05%)	Gibco	Cat#25300-054
1X DPBS	Gibco	Cat#14190-144
Keratinocyte serum free media kit	Gibco	Cat#17005-042
L-Glutamine	Gibco	Cat#25030-149
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15070063
Charcoal stripped FBS	Gibco	Cat#12676029

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the corresponding author Nupam P. Mahajan ([Nupam.Mahajan@Moffitt.org](mailto:Nupam.Mahajan@ Moffitt.org)).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Cell Lines**

RWPE-1, LNCaP, VCaP, PC3 and DU145 cells were obtained from ATCC. LAPC4 cells were obtained from Dr. Robert Reiter's Lab in UCLA and grown in IMDM media supplemented with 10% FBS. LNCaP-C4-2B cells were obtained from Dr. Evan Keller, University of Michigan. LNCaP, PC3 and DU145 cell lines were grown in RPMI 1640 supplemented with 10% FBS (Invitrogen). VCaP cells were grown in DMEM with 10% FBS (Invitrogen). RWPE-1 (ATCC) was grown in Keratinocyte serum-free media with L-glutamine (Invitrogen) supplemented with 2.5 mg EGF (Invitrogen) and 25 mg Bovine Pituitary Extract (Invitrogen). LNCaP-C4-2B cells were grown in Complete T-medium containing per Liter: 150 ml of F12, 750 ml of DMEM low glucose, 2 ml of insulin (2.5 mg/ml stock soln. = 500X), 2 ml of T3 (triiodo-thyronine, 6.825 ng/ml stock solution in 0.1% BSA in PBS = 500X), 2 ml of apo-transferin (2.5 mg/ml = 500X), 2 ml of d-Biotin (1.22 mg/10 ml 0.1% BSA in PBS + NaOH = 500X), 4 ml of Adenine Hemi-Sulfate (12.5 mg/ml in 0.1% BSA in PBS), 10 ml of Pen-Strep, 100 ml FBS. All cultures were maintained with 50 units/ml of penicillin/streptomycin (Invitrogen) and cultured in 5% CO₂ incubator.

Mouse Xenograft Studies

All animal experiments were performed using the standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice breeding and colony maintenance was performed according to IACUC protocols approved in writing by University of South Florida (USF) Division of Research Integrity and Compliance. 2x10⁶ LNCaP-C4-2B cells were suspended in 200 µl of PBS with 50% matrigel (BD Biosciences) and were implanted subcutaneously into the dorsal flank of castrated six week old male SCID C.B17 mice. Once the tumors reach approximately 100 mm³ in size (about 4-5 weeks), mice were injected subcutaneously with (R)-9bMS (or 10% DMSO in PBS as vehicle) at the concentrations 50 mg/kg of body weight, six times a week, for 4-5 weeks. Tumor volumes were measured twice weekly using calipers. Formation of tumors was monitored over an entire 10 week period. At the end of the study, all mice were humanely euthanized, tumors extracted and weighed. Additionally, spleen, liver and spleen were harvested and stained with Hematoxylin and Eosin (H and E).

For LNCaP-caACK xenograft study, similar strategy was used except that the cells were injected in male nude mice.

Prostate TMA

We used the prostate TMA built using prostate biopsies that were de-identified. This study is exempt from IRB approval as no personal information about patients is sought.

METHOD DETAILS

Proliferation Assay

For experiments involving RNA extraction or ChIP, VCaP, LAPC4 and LNCaP cells were grown in media containing 5% charcoal-stripped serum for 24–48 hr followed by treatment with 3.5, 5 or 7 μ M (*R*)-9bMS, 5 μ M enzalutamide or stimulated with 10 nM DHT for 16 hr. LNCaP-C4-2B cells were grown in its own media. To assess the effect of various treatments on cell proliferation, the cells were treated with 0.125 to 7.5 μ M of (*R*)-9bMS or enzalutamide for 96 hr and number of viable cells counted by trypan blue exclusion assay.

In Vitro Kinase Assay

For the in vitro kinase assay, 250 ng of purified ACK1 and 1 μ g of H2A/H2B/H3/H4 (New England Biolab) were incubated in the presence or absence of ACK1 inhibitor in kinase assay buffer containing 50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 10 mM DTT and 0.1 mM ATP at 30°C. After 60 min, the reaction was separated on SDS-PAGE followed by immunoblotting with pY88-H4, total H2B, H2A, H3, H4 and ACK1 antibodies.

Peptide Pull Down Assay

Two human histone H4 peptides spanning amino acids 78–92 were synthesized with Tyr88 at middle of the peptide. The sequences are as follows:

H4 (78–92): RKTVTAMDVVYALKRQG

pY88-H4 (78–92) : RKTVTAMDVVpYALKRQG

Both the peptides were biotinylated at C-terminus and immobilized on streptavidin-sepharose beads. The beads were incubated with cell lysates made from LAPC4 cells in low-salt receptor lysis buffer (LS-RLB) containing 20 mM HEPES (pH 7.5), 225 mM NaCl, 1% Triton X-100, 1 mM DTT, 10% glycerol, phosphatase inhibitors (50 mM NaF, 1 mM Na_2VO_4), and protease inhibitor mix (Roche). The beads were extensively washed with LS-RLB buffer and bound WDR5 was resolved by SDS-PAGE followed by immunoblotting with respective antibodies.

Western Blot Analysis

LNCaP, VCaP, LAPC4, LNCaP-C4-2B or PC3 cells were grown in T-75 flasks at 5×10^6 cells/ml. For AR, AR-V7, ACK1, pACK1, WDR5, H3K4me1, H3K4me2 and H3K4me3, MLL2, Actin, histones H2a, H2B, H3 and H4 and FLAG detection, treated cells were harvested and lysed by sonication in receptor lysis buffer (RLB) containing 20 mM HEPES (pH 7.5), 500 mM NaCl, 1% Triton X-100, 1 mM DTT, 10% glycerol, phosphatase inhibitors (50 mM NaF, 1 mM Na_2VO_4), and protease inhibitor mix (Roche). Lysates were quantitated and 20 to 50 mg of protein lysates were boiled in SDS sample buffer, size fractionated by SDS-PAGE, and transferred onto a PVDF membrane (Immobilon). After blocking in 5% nonfat dry milk (or 3% BSA), membranes were incubated with the following primary antibodies: AR mouse monoclonal antibody (1:3000), AR-V7 antibody (1:1000), ACK1 mouse monoclonal antibody (1:1000), pACK1 mouse monoclonal antibody (1:1000), WDR5 mouse monoclonal antibody (1:1000), H3K4me1, H3K4me2 and H3K4me3 mouse monoclonal antibody (1:1000), MLL2 mouse monoclonal antibody (1:1000), actin mouse monoclonal antibody (1:10,000), histone mouse monoclonal antibody (1:3000) or FLAG mouse monoclonal antibody (1:4000). Following three washes in PBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary antibody. The blots were washed thrice and the signals visualized by enhanced chemiluminescence (ECL) system according to manufacturer's instructions (GE Healthcare).

For ligand mediated histone H4 Y88-phosphorylation detection, cells were grown in absence of growth factors (-FBS) overnight. Cells were treated with IGF (LNCaP and LNCaP-C4-2B cells), EGF or PDGF (LAPC4) for indicated time points followed by harvesting. Cells were lysed by sonication in RLB, the lysates were quantitated and 0.5 to 1 mg of protein lysate was immunoprecipitated using 3–4 μ g of pY88-H4 antibody coupled with protein A/G-sepharose (Santacruz) overnight, followed by washes with RLB and PBS buffers. The beads were boiled in sample buffer and immunoblotting was performed using anti-H4 antibody as described above. For detection of phospho-ACK1, immunoprecipitation was performed with 2 μ g of ACK1 antibody followed by immunoblotting with pTyr antibody wherein after blocking in 3% BSA, membranes were incubated with anti-pTyr mouse monoclonal antibody (1:1000) with the rest of the procedure as described above.

Peptide Synthesis

Synthetic peptides (including biotin conjugated peptides) were custom-synthesized and HPLC-purified by Moffitt Proteomics Facility. HPLC-MS was used to confirm 95% or higher purity for each peptide. Peptides were dissolved in sterile water and aliquots were stored in -20°C.

Mass Spectrometry

Histones were purified from frozen human CRPC samples using Histone Purification Kit (Active Motif). Samples were digested overnight with modified sequencing grade trypsin (Promega, Madison, WI), Glu-C (Worthington, Lakewood, NJ), or Arg-C (Roche, Switzerland). Phosphopeptides were enriched using Phospho Select IMAC resins (Sigma). A nanoflow ultra high performance liquid chromatograph (RSLC, Dionex, Sunnyvale, CA) coupled to an electrospray bench top orbitrap mass spectrometer (Q-Exactive plus,

Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. The sample was first loaded onto a pre-column (2 cm x 100 μ m ID packed with C18 reversed-phase resin, 5 μ m, 100Å) and washed for 8 minutes with aqueous 2% acetonitrile and 0.04% trifluoroacetic acid. The trapped peptides were eluted onto the analytical column, (C18, 75 μ m ID x 50 cm, 2 μ m, 100Å, Dionex, Sunnyvale, CA). The 90-minute gradient was programmed as: 95% solvent A (2% acetonitrile + 0.1% formic acid) for 8 minutes, solvent B (90% acetonitrile + 0.1% formic acid) from 5% to 38.5% in 60 minutes, then solvent B from 50% to 90% B in 7 minutes and held at 90% for 5 minutes, followed by solvent B from 90% to 5% in 1 minute and re-equilibrate for 10 min.

The flow rate on analytical column was 300 nL/min. Sixteen tandem mass spectra were collected in a data-dependent manner following each survey scan. Both MS and MS/MS scans were performed in Orbitrap to obtain accurate mass measurement using 60 second exclusion for previously sampled peptide peaks. Sequences were assigned using Sequest (Thermo) and Mascot (www.matrixscience.com) database searches against SwissProt protein entries of the appropriate species. Oxidized methionine, carbamidomethyl cysteine, and phosphorylated serine, threonine and tyrosine were selected as variable modifications, and as many as 3 missed cleavages were allowed. The precursor mass tolerance was 20 ppm and MS/MS mass tolerance was 0.05 Da. Assignments were manually verified by inspection of the tandem mass spectra and coalesced into Scaffold reports (www.proteomesoftware.com). ArgC digested peptide KTVTAMDVVYALKR was observed as triply charged, with m/z 558.9605, which represents a mass error of 1.2 ppm; MS/MS spectrum was identified using Mascot with a score of 32.1 in CRPC sample#1. Label-Free Quantification of phospho-Tyrosine 88 in human histone H4 in CRPC samples.

Chromatin Immunoprecipitation (ChIP) and ChIP-Sequencing

C4-2B cells (5×10^7 cells) were either untreated or treated with vehicle or (R)-9bMS. Cells were harvested, fixed in p-formaldehyde and nuclei were prepared. For native ChIP (Mahajan et al., 2012b), C4-2B cells (5×10^7 cells) were treated with (R)-9bMS, harvested and nuclei were prepared. Purified nuclei were resuspended in RLB buffer and sonicated for 25 seconds (Branson sonicator). The soluble chromatin was incubated overnight at 4°C with antibodies and protein-G and A magnetic beads. The soluble chromatin was processed in the same way without immunoprecipitation and termed input DNA. The amount of immunoprecipitated DNA was determined by real-time PCR. The complexes were washed with RLB buffer followed by ChIP buffer 1 and 2 (Active Motif), eluted with elution buffer and subjected to proteinase-K treatment. Fixed ChIP DNA was 'reversed' cross linked and subjected to proteinase-K treatment. ChIP DNA was purified using PCR DNA purification columns (Qiagen).

C4-2B cells were treated with IGF ligand and either untreated or treated with (R)-9bMS. ChIP was performed using pY88-H4 antibody or IgG and DNAs were subjected to sequencing. Chromatin immunoprecipitation sequencing (ChIP-seq) was performed by the Molecular Genomics Core Facility at the Moffitt Cancer Center. Fifty nanograms of immunoprecipitated DNA was fragmented to 300 base pairs using a Covaris M220 Focused-ultrasonicator (Covaris, Inc., Woburn, MA) and then used to generate sequencing libraries using the Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA). The size and quality of the library was evaluated using the Agilent BioAnalyzer, and the library was quantitated with the Kapa Library Quantification Kit. Each enriched DNA library was then sequenced on an Illumina NextSeq 500 sequencer. The sequencing yield was very good, with over 90 million reads in each sample, of which 82.8 (IGF) and 77.9 million (IgG), respectively, mapped uniquely to the human genome GRCh37.

Flow Cytometry Analysis

$1-2 \times 10^6$ cells were seeded in 100 mM dishes. After treatment the media was aspirated, and the dishes were washed in 5 ml of 1X Phosphate Buffered Saline (PBS) via gentle rocking. After aspiration of the 1X PBS, 3 ml of Trypsin EDTA was added. Cells were harvested, washed in 1X PBS and pellet was stored in citrate storage buffer. Cells were trypsinized in 450 μ l of trypsin solution at room temperature for 10 minutes, incubated in 375 μ l trypsin inhibitor/RNase A (Sigma) solution for 10 min and stained with 250 μ l of ice-cold propidium iodide solution for 10 min and kept in dark. Samples were analyzed using the FACS Calibur flow cytometer; 10,000 events were collected and cell cycle analysis was carried out using the ModFit program.

Immunofluorescence Analysis

To visualize the intracellular distribution of pY88-H4, C42B prostate cancer cells were seeded on coverslips, transfected with HA-tagged ACK1 or vector. The cells were washed gently with 1X phosphate buffered saline (PBS) and treated with 0.5% TritonX-100 in 1X PBS for 8 min without rocking, followed by incubation in blocking buffer (3% BSA, 5% FBS in 1XPBS) for 1hr. Next, the cells were incubated with the primary antibodies, HA mouse monoclonal and H4 pY88 rabbit polyclonal at a dilution of 1:100 in blocking buffer for 1 hr. The excess antibody was washed off with 1X PBS. The cells were incubated with Alexafluor 488 conjugated anti-rabbit secondary antibody (1:500 dilution) and Alexa Fluor 647 conjugated anti-mouse secondary antibody (1:500 dilution) for 30 min. The cells were washed and coverslips were mounted on glass slides in DAPI (Vector Labs) containing mounting media. The cells were visualized using Axiovert Leica Microscope with a 40X oil immersion objective. Axiovision version 4.8 software suite was used to acquire and process images.

CRISPR-CAS9 Mediated Knockdown of ACK1 or AREMs

For ACK1 knockdown, CRISPR-CAS9 gene editing constructs were obtained from SantaCruz, while for AREM1 and AREM2 deletion, the constructs were custom synthesized by Sigma-Aldrich. PC cells were electroporated with constructs and positive cells were selected based on RFP expression using flow-sorting.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative RT-PCR and ChIP-qPCR

All RT reactions were done at the same time so that the same reactions could be used for all gene studies. For the construction of standard curves, serial dilutions of pooled sample RNA were used (50, 10, 2, 0.4, 0.08, and 0.016 ng) per reverse transcriptase reaction. One “no RNA” control and one “no Reverse Transcriptase” control were included for the standard curve. Three reactions were performed for each sample: 10 ng, 0.8 ng, and a NoRT (10 ng) control. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All standards, the no template control (H₂O), the No RNA control, the no Reverse Transcriptase control, and the no amplification control (Bluescript plasmid) were tested in six wells per gene (2 wells/plate x 3 plates/gene). All samples were tested in triplicate wells each for the 10 ng and 0.8 ng concentrations. The no RT controls were tested in duplicate wells. PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) using 2 μ l of cDNA (or ChIP DNA) and the primers in a 20 μ l final reaction mixture. After 2 min incubation at 50°C, AmpliTaq Gold was activated by 10 min incubation at 95°C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C and hybridization of primers for 1 min at 55°C. Dissociation curves were generated for each plate to verify the integrity of the primers. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The actin data were used for normalizing the gene values.

Gene Expression Array Analysis

C4-2B, VCaP, LAPC4, LNCaP were grown in androgen-deprived media and were treated with 3.5 μ M (*R*)-9bMS for 24 hr and total RNA was extracted using RNeasy Mini Kit (Qiagen) for gene expression array analysis. Expression profiling was performed using the Agilent Whole Human Genome Four-Plex 44K Microarray according to the manufacturer’s protocol. All samples were run in technical duplicates or quadruplicates against control. Differential gene expression was analyzed using Nexus expression 3.0 software. Over- and under- expressed gene sets were generated by filtering to include only data points that showed either over- or underexpression by at least two-folds (log ratio with $p < 0.001$) in all hybridizations. GSEA was performed using the JAVA program (<http://www.broadinstitute.org/gsea>) as described previously (Subramanian et al., 2005). Specifically, genes were ranked based on log fold change and pre-ranked setting was used to perform the analyses.

Computational Analysis of ChIP-Seq Data: Sequence Analysis

The 75-nt paired end sequence reads were mapped to the genome using the BWA-MEM algorithm. Alignment information for each read was stored in the output file *.bam. Only reads that mapped uniquely with proper pairing were used in the subsequent analysis.

Determination of Fragment Density

Because the 5’ ends of the sequence reads represent the end of the ChIP or immunoprecipitation fragments, the reads were extended in silico (using MAC2) at their 3’ ends to a length of 173–244 bp, based on the fragment length calculated from the read pairs.

Peak Finding

Peak regions were called using the MACS2 software with the following options -f BAMPE -SPMR -q 0.01 -broad. The “BAMPE” option was used for calculating fragment lengths from the paired end reads, “SPMR” for normalizing read depths to number of fragments per million reads, “broad” for compositing broad regions from nearby peak regions, and the q value (FDR) cutoff was set to 0.01. A total of 370 peak regions were called between the IGF sample and the IgG control.

Motif Analysis

HOMER (v4.7, 8-25-2014) program (<http://homer.salk.edu/homer/>) was used to identify de novo motifs in pY88-H4 ChIP-seq data.

Tissue Microarray (TMA) Analysis

For assessment of pY88-H4, H3K4me3 and AR expression in prostate cancer, immunohistochemistry was carried out on a high-density prostate TMAs (n = 250 cores) containing samples of different stages of disease as described earlier (Mahajan et al., 2010b).

Statistical Analysis

The Mantel-Haenszel χ^2 test was performed to examine if there is an increasing trend for H3K4me3 and AR with respect to different progression stages of prostate cancer. The ordinal intensity levels of H3K4me3 and AR 0, 1, 2, 3, 4, 6, 9 were pooled into 6 levels (as 0, 1, 2, 3, 4, and 6 and above) to accommodate the rare observations in the highest intensity level in most stages. Analysis of variance was performed to examine whether the expression levels of H3K4me3 and AR differ among different tumor stages. Boxplots were used to summarize the intensity distribution at each progression stage. Furthermore, Tukey-Kramer method was performed to examine between which pairs of stages the expression levels are different. This post-hoc procedure adjusts for all pairwise comparisons and simultaneous inference. When more than one sample was obtained from a patient, the intensity of the most progressed stage was used for the analysis. Correlation between H3K4me3 and AR was explored using Spearman ranked correlation analysis. Statistical differences between the groups were determined using log-rank test.

Mass Spectrometry Data Analysis

Xcalibur software (Thermo) was used to extract ion chromatogram of H4 pY88-containing Arg-C digested peptide KTVTAMDVVYALKR. m/z tolerance was set to ± 0.02 Th and retention time (RT) tolerance was set to ± 60 seconds. Area under the curve (AUC) values were used to quantify relative intensities between samples.

DATA AND SOFTWARE AVAILABILITY**Links to GEP Database**

The ChIP-Seq as well as microarray data has been submitted to GEO database, the accession numbers are GSE79689 and GSE79658, respectively.

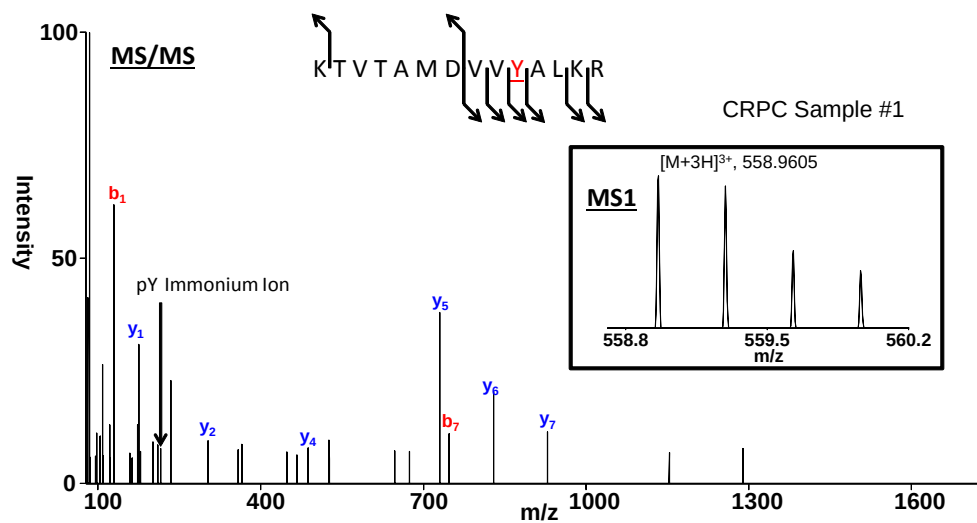
Supplemental Information

ACK1/TNK2 Regulates Histone H4

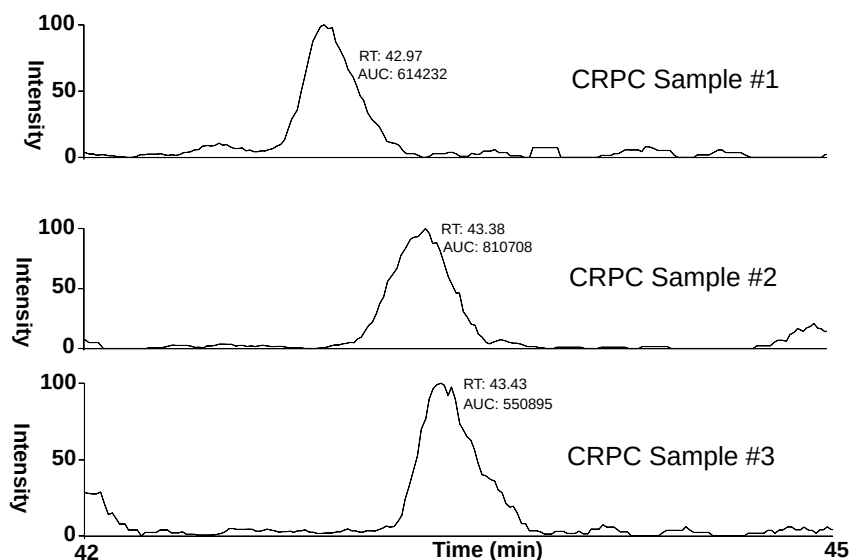
**Tyr88-phosphorylation and AR Gene Expression
in Castration-Resistant Prostate Cancer**

Kiran Mahajan, Pavani Malla, Harshani R. Lawrence, Zhihua Chen, Chandan K. Sinha, Rohit Malik, Sudhanshu Shukla, Jongphil Kim, Domenico Coppola, Nicholas J. Lawrence, and Nupam P. Mahajan

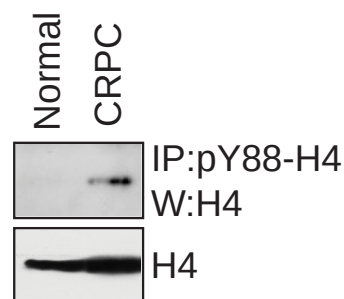
A



B



C



D

80	TVTAMDVVYALKRQGRTLYG	<i>H. sapiens</i>
80	TVTAMDVVYALKRQGRTLYG	<i>B. taurus</i>
80	TVTAMDVVYALKRQGRTLYG	<i>M. musculus</i>
80	TVTAMDVVYALKRQGRTLYG	<i>R. norvegicus</i>
80	TVTAMDVVYALKRQGRTLYG	<i>X. laevis</i>
80	TVTAMDVVYALKRQGRTLYG	<i>D. rerio</i>
80	TVTAMDVVYALKRQGRTLYG	<i>B. mori</i>
80	TVTAMDVVYALKRQGRTLYG	<i>D. melanogaster</i>
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80	TVTSLDVVYALKRQGRTLYG	<i>S. cerevisiae</i>

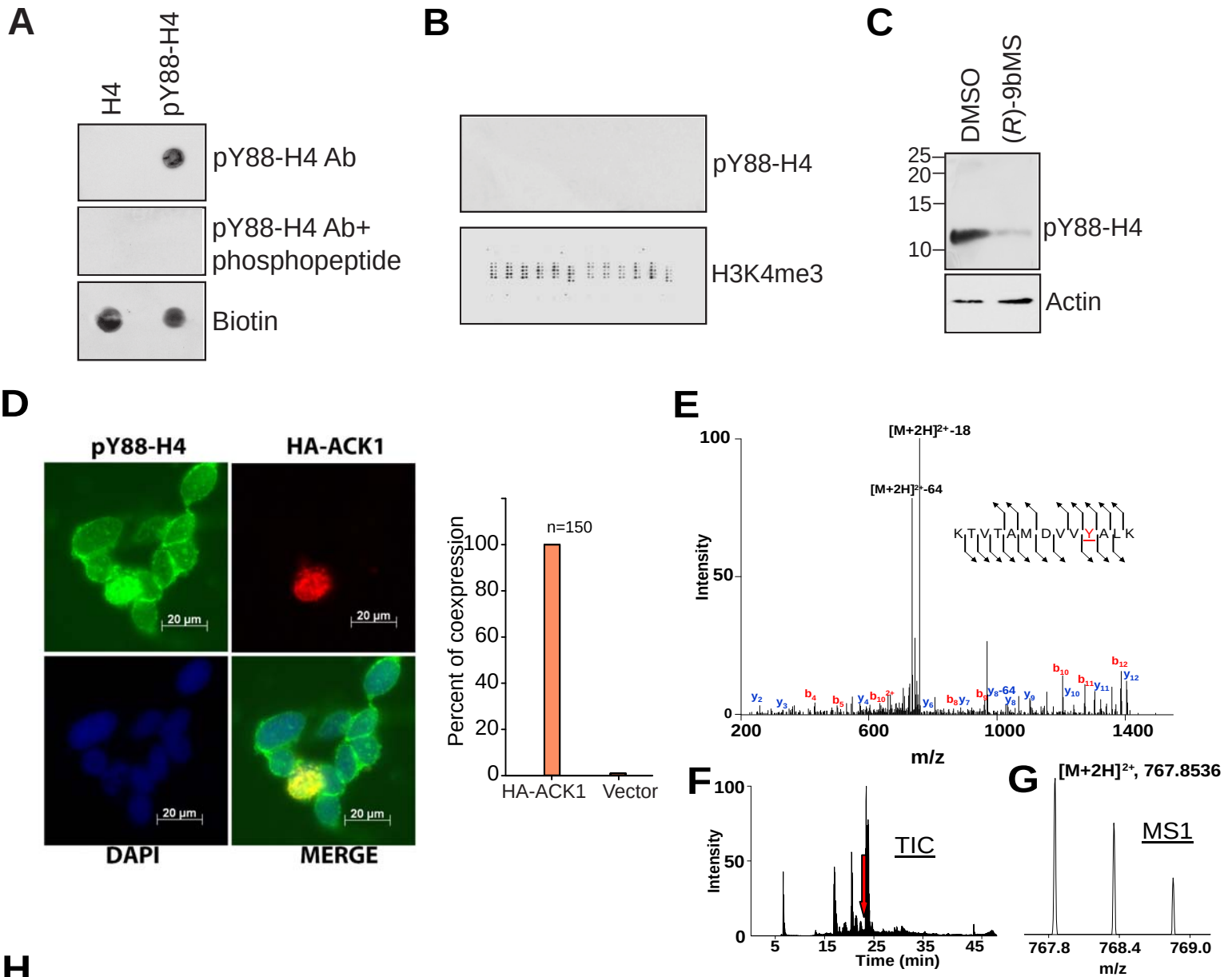
Figure S1 (related to Figure 1). Detection of histone H4 Y88-phosphorylation in human CRPCs

(A) Histones purified from 3 freshly frozen human CRPC samples were processed for LC-MS/MS analysis. ArgC digested peptide KTVTAMDVVYALKR was observed as triply charged, with m/z 558.9605, which represents a mass error of 1.2 ppm; MS/MS spectrum was identified using Mascot with a score of 32.1 in CRPC sample#1.

(B) Label-Free quantification of phospho-Tyrosine 88 in human histone H4 in CRPC samples #1-3. Xcalibur software (Thermo) was used to extract the ion chromatogram of H4 pY88 containing Arg-C digested peptide KTVTAMDVVYALKR. m/z tolerance was set to ± 0.02 Th and retention time (RT) tolerance was set to ± 60 seconds. Area under the curve (AUC) values were used to quantify the relative intensities between samples.

(C) Total histones isolated from a freshly frozen human CRPC sample#1 and a normal prostate sample were immunoprecipitated with pY88-H4 antibody, followed by immunoblotting with H4 antibody.

(D) Evolutionary conservation of Tyr88 in histone H4.



H

ACK1_HUMAN (100%), 114,569.8 Da
Activated CDC42 kinase 1 OS=Homo sapiens GN=TNK2 PE=1 SV=3

MQPEEGTGWL	LELLSEVQLQ	QYFLRLRDDL	NVTRLSHFEY	VKNEDLEKIG	MGRPGQRRLLW	EAVKRRKALC	KRKSWMSKVF	SGKRLEAEFP
PHHSQSTFRK	TSPAPGGPAG	EGPLQSLTCL	IGEKDLRLLE	KLGDGSGFGV	RRGEWDAPSG	KTYSVAVKCL	KPDVLSQPEA	MDDFFIREVNA
MHSLDHRNLI	RLYGVVLTTP	MKMVTLEAPL	GSLDLRLRKH	QGHFLLGLTL	RYAVQVAEGM	GYLESKRFIH	RDLAARNLLI	ATRDLVKIGD
FGLMRALPON	DDHYVMQEHF	KVPFAWCAPE	SLKTRTFSHA	SOTWMFGVTL	WEMFTYGEQP	WIGLNGSQIL	HKIDKEGERL	PRPEDCPQDI
YNVMVQCWAH	KPEDRPTFVA	LKDFLLLEAQP	TDMRALQDFE	EPDKLHIQMN	DVITVIEGRA	ENYWWRGQNT	RTLGVGPFPR	NVVTSVAGLS
AQDISQPLQN	SFIHTGHGDS	DRHHCWGFDP	RIDELYLGNP	MDPPDLLSVE	LSTSRPPQHL	GGVKKPTYDP	VSEDDQDPLSS	DFKRLGLRKP
GLPRGLWLAK	PSARVPGTKA	SRGSGAEVTL	IDFGEERPVP	ALRPCAPSLA	QLAMDACSLL	DETTPQSPTR	ALPRPLHPTP	VVDWDARPLP
GLPAYDDVAQ	DEDDDFEICS	NSTLVGAGVP	AGPSQGGQTN	AFVPEQARPP	PPLEDNLFLL	PQGGGKPPSP	AQTAEIFQAL	QQECMRQLQA
PAGSPAPSPS	PGGDDKQVVP	PRVDPIDPRPT	RPHVQLSPAP	PGEETSQWP	GPAASPRVPP	REPLSPQGS	TSPSLVPPGS	SPLPPRLSSS
PGKTMPTTQS	FASDPKATP	QVIOAPGPR	GPCILPIVRD	GKKVSSSTHY	LLPERPSYLE	RYQRFLREAQ	SPEEPTPLPV	PLLLPPPSTP
APAAPATATVR	PMPQAAIDPK	ANFSTNNNSNP	GARPPLPRAT	ARKLPQRCPPG	DGPEAGR PAD	KIQMAMVHGV	TTEECQAALQ	CHGWSVQRAA
QYLKVEQLFG	LGLRPRGECH	KVLEMFDFNL	EQAGCHLLGS	WGPAAHKKR				

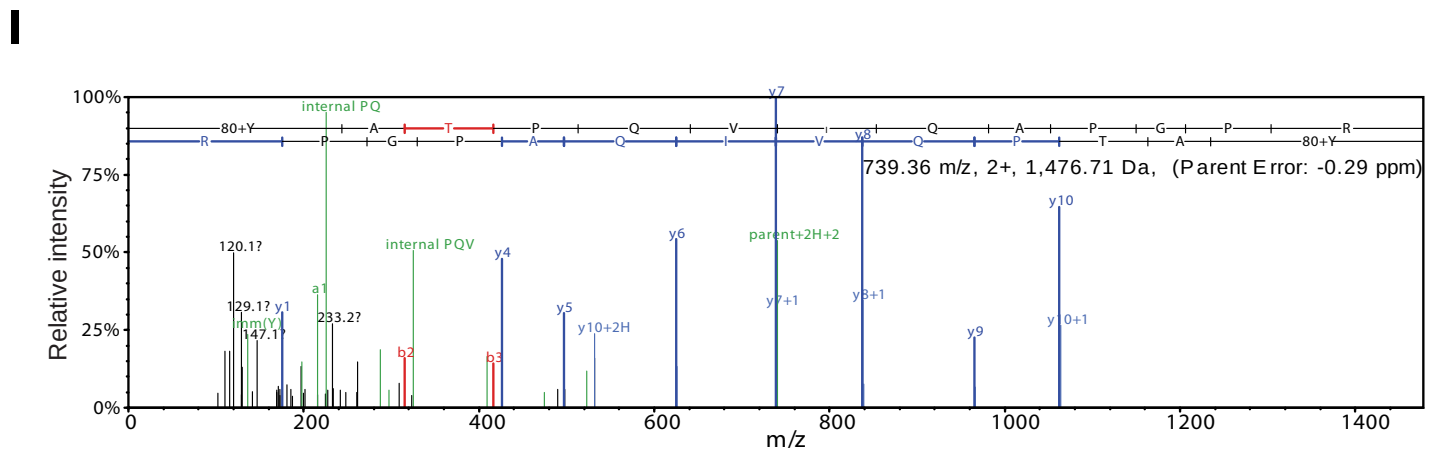


Figure S2 (related to Figure 1). ACK1 phosphorylates the histone H4 at tyrosine 88

(A) Biotinylated histone peptides were immunoblotted with indicated antibody.

(B) The MODified Histone Peptide Array (Active Motif) containing 384 unique histone modification combinations was immunoblotted with pY88-H4 antibody or H3K4me3 antibody (as control).

(C) C4-2B cells treated with IGF or IGF+(R)-9bMS were immunoblotted for pY88-H4.

(D) C4-2B cells transfected with HA-tagged ACK1 construct, followed by immunofluorescence staining with HA (mouse monoclonal) and pY88-H4 (rabbit polyclonal) antibodies. Scale bar 20 μ m.

(E-G) Histones purified from HEK293T cells transfected with ACK1, were digested with trypsin and subjected to LC-MS/MS analysis. The H4-Y88 peptide was detected at 23 minutes in the total ion chromatogram (E) with a mass-to-charge ratio of 767.8 (F). Tandem mass spectrum matched sequence is shown (G).

(H) HEK293T cells transfected with MYC-tagged H4 expressing construct were affinity purified with MYC beads and subjected to mass spectrometry. ACK1 peptides identified by mass spectrometry are highlighted.

(I) The tandem mass spectrum revealing ACK1 phosphorylation at Tyr827 corresponds to the peptide YATPQVIQAPGPR.

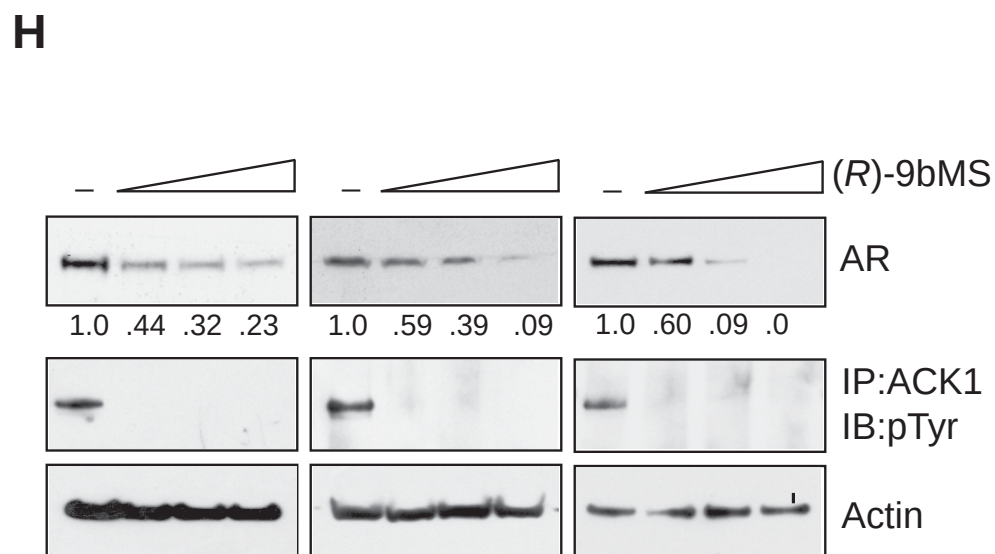
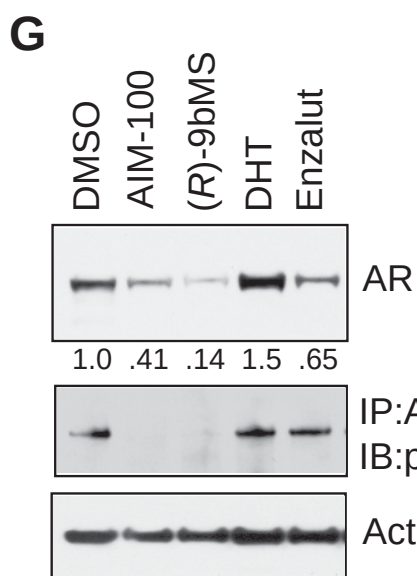
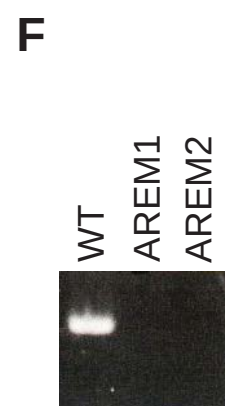
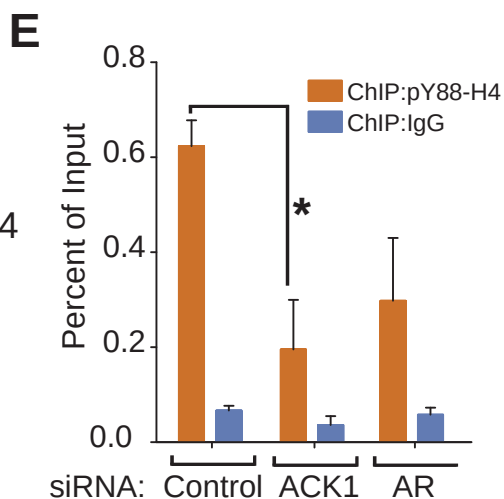
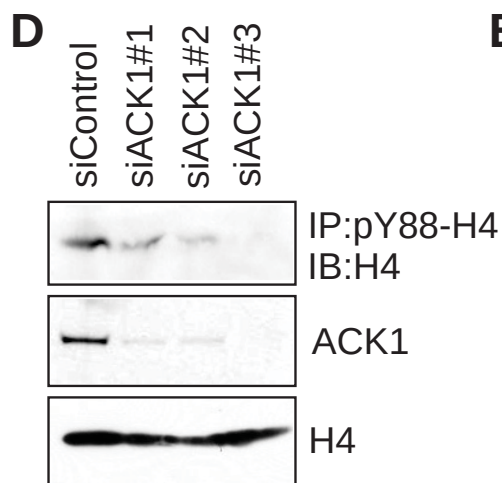
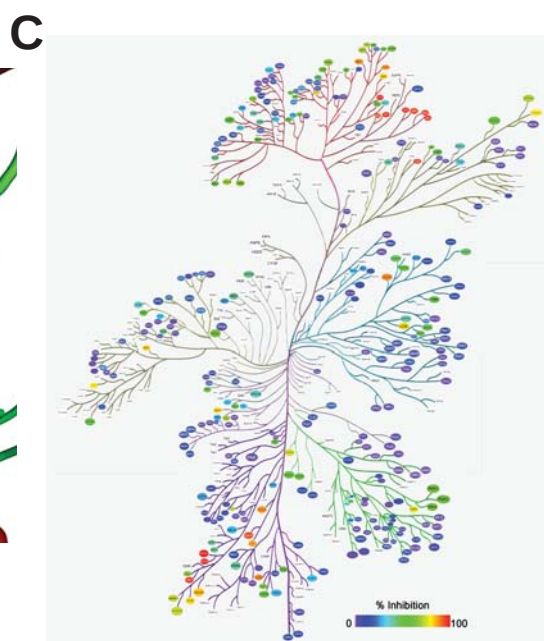
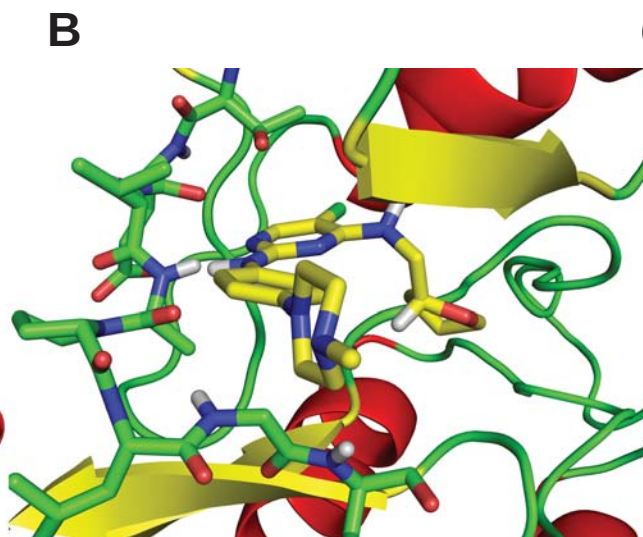
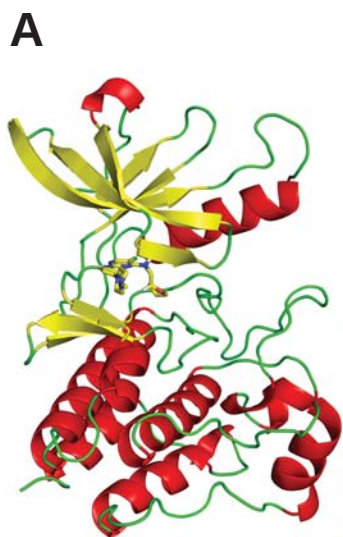


Figure S3 (related to Figures 1, 2 and 3). AR expression in androgen-deficient environment is dependent on ACK1

(A) Compound (*R*)-9b (yellow) docked to ACK1 (pdb 4EWH), with ACK1 shown in ribbon representation with helices shown in red, β -strands shown as yellow arrows and loops shown in green.

(B) Expanded stick model of (*R*)-9b (yellow) docked to the ATP binding site of ACK1 (pdb 4EWH), showing the amino acid residues of the kinase hinge in stick representation.

(C) Small molecule/kinase interaction maps for (*R*)-9b. The kinase profiling was done at 250 nM resulting in inhibition of ACK1 and 11 other kinases to >90%. The kinases that bind strongly to (*R*)-9b are marked with red.

(D) Androgen-deprived LAPC4 cells electroporated with control or three distinct ACK1 siRNAs, were stimulated with EGF for 20 min. Lysates were immunoprecipitated with pY88-H4 antibody followed by immunoblotting with H4 antibody.

(E) C4-2B cells transfected with control, ACK1 and AR siRNA and ChIP was performed with pY88-H4 antibody, followed by qPCR using primers corresponding to AREM1. Data are represented as mean $\pm$ SEM ($n = 2$, three replicates). * $p < 0.05$.

(F) C4-2B cells transfected with RFP-tagged CRISPR-Cas9 construct targeting AREM1 and AREM2 were selected for RFP positive cells by flow cytometry. Genomic DNA was purified and PCR was performed with AREM1 and AREM2 specific primers.

(G) Androgen deprived LAPC4 cells were treated with vehicle or ACK1 inhibitors, (*R*)-9bMS or AIM-100 (5 μ M, 36 hr), enzalutamide (5 μ M, 36 hr), DHT (10 nM, 16 hr), followed by immunoblotting with AR, pACK1 or actin antibody. Relative quantitation of AR expression is shown below the top panel.

(H) Androgen deprived LAPC4 (left), LNCaP (middle) and VCaP (right) cells were untreated or treated with (*R*)-9bMS (1, 2.5 and 7 μ M, 36 hr) followed by immunoblotting with AR or actin antibodies. Lysates were also immunoprecipitated with ACK1 antibody followed by pTyr antibody. Relative quantitation of AR expression is shown below top panel.

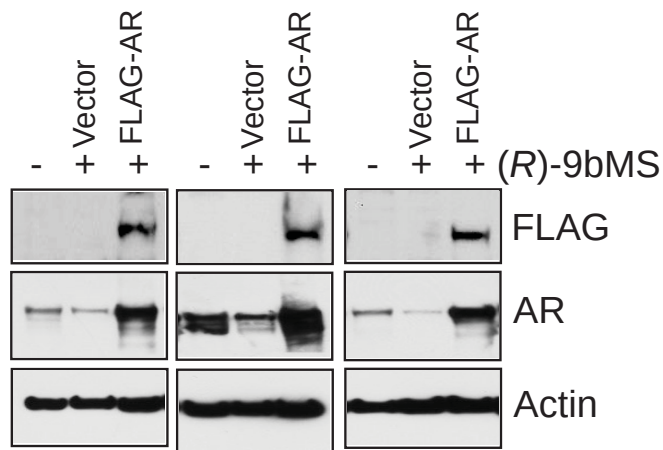
Table S1 (related to Figure 1). H4-interacting proteins identified using mass spectrometry

Proteins	Accession Number	Mol. Wt.	Uniq Peptides
Cluster of Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SACTN4_HUMAN [3]		105 kDa	91
Spectrin alpha chain, non-erythrocytic 1 OS=Homo sapiens GN=SPTN1_HUMAN		285 kDa	112
Drebrin OS=Homo sapiens GN=DBN1 PE=1 SV=4	DREB_HUMAN	71 kDa	32
Cytospin-A OS=Homo sapiens GN=SPECC1L PE=1 SV=2	CYTSA_HUMAN	125 kDa	49
Cingulin OS=Homo sapiens GN=CGN PE=1 SV=2	CING_HUMAN	136 kDa	54
LIM domain and actin-binding protein 1 OS=Homo sapiens GN=LIMA1_HUMAN		85 kDa	40
Angiomotin OS=Homo sapiens GN=AMOT PE=1 SV=1	AMOT_HUMAN	118 kDa	46
Poly [ADP-ribose] polymerase 1 OS=Homo sapiens GN=PARP1_HUMAN		113 kDa	42
Cluster of Neurofilament medium polypeptide OS=Homo sapiens GN=NFM_HUMAN [2]		102 kDa	44
Protein phosphatase 1 regulatory subunit 12A OS=Homo sapiens GN=MYPT1_HUMAN		115 kDa	43
Cluster of Heat shock protein HSP 90-beta OS=Homo sapiens GN=HS90B_HUMAN [3]		83 kDa	41
Leucine zipper protein 1 OS=Homo sapiens GN=LUZP1 PE=1 SV=1	LUZP1_HUMAN	120 kDa	41
Insulin receptor substrate 4 OS=Homo sapiens GN=IRS4 PE=1 SV=1	IRS4_HUMAN	134 kDa	37
Neurabin-2 OS=Homo sapiens GN=PPP1R9B PE=1 SV=2	NEB2_HUMAN	89 kDa	33
Protein flightless-1 homolog OS=Homo sapiens GN=FLII PE=1 SV=1	FLII_HUMAN	145 kDa	40
Activated CDC42 kinase 1 OS=Homo sapiens GN=TNK2 PE=1 ACK1_HUMAN		115 kDa	28
Desmoglein-2 OS=Homo sapiens GN=DSG2 PE=1 SV=2	DSG2_HUMAN	122 kDa	36
Band 4.1-like protein 2 OS=Homo sapiens GN=EPB41L2 PE=1 SV=1	EPB41L2_HUMAN	113 kDa	37
Programmed cell death 6-interacting protein OS=Homo sapiens GN=PDC6I_HUMAN		96 kDa	34
Cluster of Catenin alpha-1 OS=Homo sapiens GN=CTNNA1 PE=1 SV=1	CTNA1_HUMAN [2]	100 kDa	34
Ankycorbin OS=Homo sapiens GN=RAI14 PE=1 SV=2	RAI14_HUMAN	110 kDa	36
Tight junction protein ZO-2 OS=Homo sapiens GN=TJP2 PE=1 SV=1	ZO2_HUMAN	134 kDa	39
Protein arginine N-methyltransferase 5 OS=Homo sapiens GN=PFANM5_HUMAN		73 kDa	27
A-kinase anchor protein 2 OS=Homo sapiens GN=AKAP2 PE=1 SV=1	AKAP2_HUMAN	95 kDa	25
ATP-dependent RNA helicase A OS=Homo sapiens GN=DHX9_HUMAN		141 kDa	33
Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3	PLAK_HUMAN	82 kDa	30
MAGUK p55 subfamily member 5 OS=Homo sapiens GN=MPP5_HUMAN	MPP5_HUMAN	77 kDa	30
X-ray repair cross-complementing protein 5 OS=Homo sapiens GN=XRCC5_HUMAN		83 kDa	24
Cluster of Dedicator of cytokinesis protein 7 OS=Homo sapiens GN=DOCK7_HUMAN		243 kDa	32
Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	54 kDa	26
Annexin A6 OS=Homo sapiens GN=ANXA6 PE=1 SV=3	ANXA6_HUMAN	76 kDa	27
Centrosomal protein of 131 kDa OS=Homo sapiens GN=CEP131_HUMAN		122 kDa	31
Cytospin-B OS=Homo sapiens GN=SPECC1 PE=1 SV=1	CYTSB_HUMAN	119 kDa	28
Cluster of Ezrin OS=Homo sapiens GN=EZR PE=1 SV=4 (EZRI_HUMAN [3])	EZRI_HUMAN [3]	69 kDa	26
Cluster of AP-2 complex subunit beta OS=Homo sapiens GN=AP2B1_HUMAN [2]		105 kDa	26
Plakophilin-2 OS=Homo sapiens GN=PKP2 PE=1 SV=2	PKP2_HUMAN	97 kDa	25
Protein 4.1 OS=Homo sapiens GN=EPB41 PE=1 SV=4	EPB41_HUMAN	97 kDa	24
Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3	CALD1_HUMAN	93 kDa	23
Nexilin OS=Homo sapiens GN=NEXN PE=1 SV=1	NEXN_HUMAN	81 kDa	23
Stress-70 protein, mitochondrial OS=Homo sapiens GN=HSPA9_HUMAN	GRP75_HUMAN	74 kDa	23
78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5_HUMAN	GRP78_HUMAN	72 kDa	24
FACT complex subunit SPT16 OS=Homo sapiens GN=SUPT16H_HUMAN	SP16H_HUMAN	120 kDa	25
Nucleolar RNA helicase 2 OS=Homo sapiens GN=DDX21_HUMAN		87 kDa	27
WD repeat-containing protein 6 OS=Homo sapiens GN=WDR6_HUMAN	WDR6_HUMAN	122 kDa	23
X-ray repair cross-complementing protein 6 OS=Homo sapiens GN=XRCC6_HUMAN		70 kDa	18

Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3	DESP_HUMAN	332 kDa	23
Src substrate cortactin OS=Homo sapiens GN=CTTN PE=1 SV=2	SRC8_HUMAN	62 kDa	17
Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC PE=1 SV=5	CLH1_HUMAN	192 kDa	25
Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4	FLNA_HUMAN	281 kDa	27
Importin-5 OS=Homo sapiens GN=IPO5 PE=1 SV=4	IPO5_HUMAN	124 kDa	21
LIM domain only protein 7 OS=Homo sapiens GN=LMO7 PE=1 S'	LMO7_HUMAN	193 kDa	22
Tight junction protein ZO-1 OS=Homo sapiens GN=TJP1 PE=1 S'	ZO1_HUMAN	195 kDa	25
Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4	EF2_HUMAN	95 kDa	20
Heterogeneous nuclear ribonucleoprotein M OS=Homo sapiens G	HNRPM_HUMAN	78 kDa	21
Leucine-rich repeat and calponin homology domain-containing prc	LRCH2_HUMAN	85 kDa	20
DNA damage-binding protein 1 OS=Homo sapiens GN=DDB1 PE	DDB1_HUMAN	127 kDa	20
Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA	HSP7C_HUMAN	71 kDa	23
Cluster of Liprin-alpha-1 OS=Homo sapiens GN=PPFIA1 PE=1 S'	LIPA1_HUMAN	136 kDa	19
Putative ATP-dependent RNA helicase DHX30 OS=Homo sapien	DHX30_HUMAN	134 kDa	21
Cluster of Interleukin enhancer-binding factor 3 OS=Homo sapien	ILF3_HUMAN [2]	95 kDa	17
Plakophilin-4 OS=Homo sapiens GN=PKP4 PE=1 SV=2	PKP4_HUMAN	132 kDa	21
Splicing factor 3B subunit 1 OS=Homo sapiens GN=SF3B1 PE=1	SF3B1_HUMAN	146 kDa	18
Catenin beta-1 OS=Homo sapiens GN=CTNNB1 PE=1 SV=1	CTNB1_HUMAN	85 kDa	22
Transitional endoplasmic reticulum ATPase OS=Homo sapiens G	ITERA_HUMAN	89 kDa	17
Cluster of Probable ATP-dependent RNA helicase DDX17 OS=Ho	DDX17_HUMAN [2]	80 kDa	16
Cadherin-2 OS=Homo sapiens GN=CDH2 PE=1 SV=4	CADH2_HUMAN	100 kDa	14
Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1	GELS_HUMAN	86 kDa	14
Pleckstrin homology-like domain family B member 2 OS=Homo sa	PHLB2_HUMAN	142 kDa	16
Isoleucine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=IAR	SYIC_HUMAN	145 kDa	17
Cluster of Cytoplasmic FMR1-interacting protein 1 OS=Homo sapi	CYFP1_HUMAN [2]	145 kDa	16
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15_HUMAN	91 kDa	16
ELKS/Rab6-interacting/CAST family member 1 OS=Homo sapien	RB612_HUMAN	128 kDa	19
Gamma-adducin OS=Homo sapiens GN=ADD3 PE=1 SV=1	ADDG_HUMAN	79 kDa	16
Band 4.1-like protein 3 OS=Homo sapiens GN=EPB41L3 PE=1 S'	E41L3_HUMAN	121 kDa	17
Leucine-rich repeat and calponin homology domain-containing prc	LRCH3_HUMAN	86 kDa	17
Neurabin-1 OS=Homo sapiens GN=PPP1R9A PE=1 SV=2	NEB1_HUMAN	123 kDa	17
Cluster of SLIT-ROBO Rho GTPase-activating protein 2 OS=Hom	SRGP2_HUMAN	121 kDa	15
Cluster of Angiomotin-like protein 1 OS=Homo sapiens GN=AMO	AMOL1_HUMAN	107 kDa	21
CD2-associated protein OS=Homo sapiens GN=CD2AP PE=1 SV	CD2AP_HUMAN	71 kDa	16
Cluster of ATP-dependent RNA helicase DDX3X OS=Homo sapie	DDX3X_HUMAN	73 kDa	15
Kinesin-like protein KIF23 OS=Homo sapiens GN=KIF23 PE=1 S'	KIF23_HUMAN	110 kDa	14
Leucine zipper putative tumor suppressor 2 OS=Homo sapiens G	LZTS2_HUMAN	73 kDa	11
Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3	NUCL_HUMAN	77 kDa	15
116 kDa U5 small nuclear ribonucleoprotein component OS=Hom	U5S1_HUMAN	109 kDa	15
Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1	HSP71_HUMAN	?	17
Kinesin-like protein KIF14 OS=Homo sapiens GN=KIF14 PE=1 S'	KIF14_HUMAN	186 kDa	14
FACT complex subunit SSRP1 OS=Homo sapiens GN=SSRP1 P	SSRP1_HUMAN	81 kDa	16
Cluster of Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1	TBB5_HUMAN [2]	50 kDa	13
ATP-citrate synthase OS=Homo sapiens GN=ACLY PE=1 SV=3	ACLY_HUMAN	121 kDa	14
Bcl-2-associated transcription factor 1 OS=Homo sapiens GN=BC	BCLF1_HUMAN	106 kDa	12
ATP-dependent RNA helicase DDX1 OS=Homo sapiens GN=DD	DDX1_HUMAN	82 kDa	15
Protein EFR3 homolog A OS=Homo sapiens GN=EFR3A PE=1 S'	EFR3A_HUMAN	93 kDa	13

Epidermal growth factor receptor substrate 15-like 1 OS=Homo sapiens GN=EP15R PE=1 SV=2	EP15R_HUMAN	94 kDa	14
Filamin-B OS=Homo sapiens GN=FLNB PE=1 SV=2	FLNB_HUMAN	278 kDa	18
Cluster of Inositol 1,4,5-trisphosphate receptor type 3 OS=Homo sapiens GN=ITPR3 PE=1 SV=1	ITPR3_HUMAN [3]	304 kDa	11
Merlin OS=Homo sapiens GN=NF2 PE=1 SV=1	MERL_HUMAN	70 kDa	13
E3 ubiquitin-protein ligase MIB1 OS=Homo sapiens GN=MIB1 PE=1 SV=1	MIB1_HUMAN	110 kDa	13
Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3	PLEC_HUMAN	532 kDa	14
Serine/threonine-protein kinase RIO1 OS=Homo sapiens GN=RIOK1 PE=1 SV=1	RIOK1_HUMAN	66 kDa	13
Epidermal growth factor receptor substrate 15 OS=Homo sapiens GN=EPS15 PE=1 SV=1	EPS15_HUMAN	99 kDa	12
Structural maintenance of chromosomes protein 2 OS=Homo sapiens GN=SMC2 PE=1 SV=1	SMC2_HUMAN	136 kDa	14
Beta-adducin OS=Homo sapiens GN=ADD2 PE=1 SV=3	ADD2_HUMAN	81 kDa	12
Heat shock protein 105 kDa OS=Homo sapiens GN=HSPH1 PE=1 SV=1	HS105_HUMAN	97 kDa	14
Nck-associated protein 1 OS=Homo sapiens GN=NCKAP1 PE=1 SV=1	NCKP1_HUMAN	129 kDa	13
Cluster of SWI/SNF-related matrix-associated actin-dependent regulator of SMCA5 OS=Homo sapiens GN=SMCA5 PE=1 SV=1	SMCA5_HUMAN [2]	122 kDa	14
Sperm-specific antigen 2 OS=Homo sapiens GN=SSFA2 PE=1 SV=1	SSFA2_HUMAN	138 kDa	11
Leucine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=LARS SYLC OS=Homo sapiens GN=SYLC PE=1 SV=1	SYLC_HUMAN	134 kDa	13
Thyroid hormone receptor-associated protein 3 OS=Homo sapiens GN=TR150 PE=1 SV=1	TR150_HUMAN	109 kDa	10
Apoptosis-stimulating of p53 protein 2 OS=Homo sapiens GN=TP53 PE=1 SV=1	ASPP2_HUMAN	126 kDa	11
Death domain-associated protein 6 OS=Homo sapiens GN=DAXX PE=1 SV=1	DAXX_HUMAN	81 kDa	12
InaD-like protein OS=Homo sapiens GN=INADL PE=1 SV=3	INADL_HUMAN	196 kDa	10
Matrin-3 OS=Homo sapiens GN=MATR3 PE=1 SV=2	MATR3_HUMAN	95 kDa	10
Unconventional myosin-XVIIIa OS=Homo sapiens GN=MYO18A PE=1 SV=1	MYO18A_HUMAN	233 kDa	11
Nuclear pore complex protein Nup93 OS=Homo sapiens GN=NUF93 PE=1 SV=1	NUP93_HUMAN	93 kDa	10
Phosphatidylinositol 3-kinase regulatory subunit beta OS=Homo sapiens GN=P85B PE=1 SV=1	P85B_HUMAN	82 kDa	15
Progesterone-induced-blocking factor 1 OS=Homo sapiens GN=PIBF1 PE=1 SV=1	PIBF1_HUMAN	90 kDa	10
Pleckstrin homology domain-containing family A member 7 OS=Homo sapiens GN=PKHA7 PE=1 SV=1	PKHA7_HUMAN	127 kDa	10
26S proteasome non-ATPase regulatory subunit 2 OS=Homo sapiens GN=PSMD2 PE=1 SV=1	PSMD2_HUMAN	100 kDa	11
Splicing factor 3B subunit 3 OS=Homo sapiens GN=SF3B3 PE=1 SV=1	SF3B3_HUMAN	136 kDa	13
Ubiquitin-like modifier-activating enzyme 1 OS=Homo sapiens GN=UBA1 PE=1 SV=1	UBA1_HUMAN	118 kDa	11
Exportin-1 OS=Homo sapiens GN=XPO1 PE=1 SV=1	XPO1_HUMAN	123 kDa	13
AP-2 complex subunit alpha-1 OS=Homo sapiens GN=AP2A1 PE=1 SV=1	AP2A1_HUMAN	108 kDa	16
AP-2 complex subunit alpha-2 OS=Homo sapiens GN=AP2A2 PE=1 SV=1	AP2A2_HUMAN	104 kDa	14
Ras GTPase-activating-like protein IQGAP1 OS=Homo sapiens GN=IQGA1 PE=1 SV=1	IQGA1_HUMAN	189 kDa	13
Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1 SV=1	K2C8_HUMAN	54 kDa	17
DNA mismatch repair protein Msh2 OS=Homo sapiens GN=MSH2 PE=1 SV=1	MSH2_HUMAN	105 kDa	10
Lysine--tRNA ligase OS=Homo sapiens GN=KARS PE=1 SV=3	SYK_HUMAN	68 kDa	11
Protein unc-45 homolog A OS=Homo sapiens GN=UNC45A PE=1 SV=1	UN45A_HUMAN	103 kDa	12
Cluster of ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25 ADT2 OS=Homo sapiens GN=ADT2 PE=1 SV=1	ADT2_HUMAN [2]	33 kDa	11
C-1-tetrahydrofolate synthase, cytoplasmic OS=Homo sapiens GN=C1TC PE=1 SV=1	C1TC_HUMAN	102 kDa	12
Clathrin interactor 1 OS=Homo sapiens GN=CLINT1 PE=1 SV=1	EPN4_HUMAN	68 kDa	11
Double-strand break repair protein MRE11A OS=Homo sapiens GN=MRE11 PE=1 SV=1	MRE11_HUMAN	81 kDa	7
Cluster of Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABP1 PE=1 SV=1	PABP1_HUMAN	71 kDa	15
Rho GTPase-activating protein 11A OS=Homo sapiens GN=ARHGAP11A PE=1 SV=1	ARHGAP11A_HUMAN	114 kDa	10
DNA-directed RNA polymerase II subunit RPB2 OS=Homo sapiens GN=RPB2 PE=1 SV=1	RPB2_HUMAN	134 kDa	11
Transcription intermediary factor 1-beta OS=Homo sapiens GN=TIF1B PE=1 SV=1	TIF1B_HUMAN	89 kDa	11
Rho guanine nucleotide exchange factor 2 OS=Homo sapiens GN=ARHG2 PE=1 SV=1	ARHG2_HUMAN	112 kDa	10
General transcription factor II-I OS=Homo sapiens GN=GTF2I PE=1 SV=1	GTF2I_HUMAN	112 kDa	8
Histone acetyltransferase type B catalytic subunit OS=Homo sapiens GN=HAT1 PE=1 SV=1	HAT1_HUMAN	50 kDa	8

A

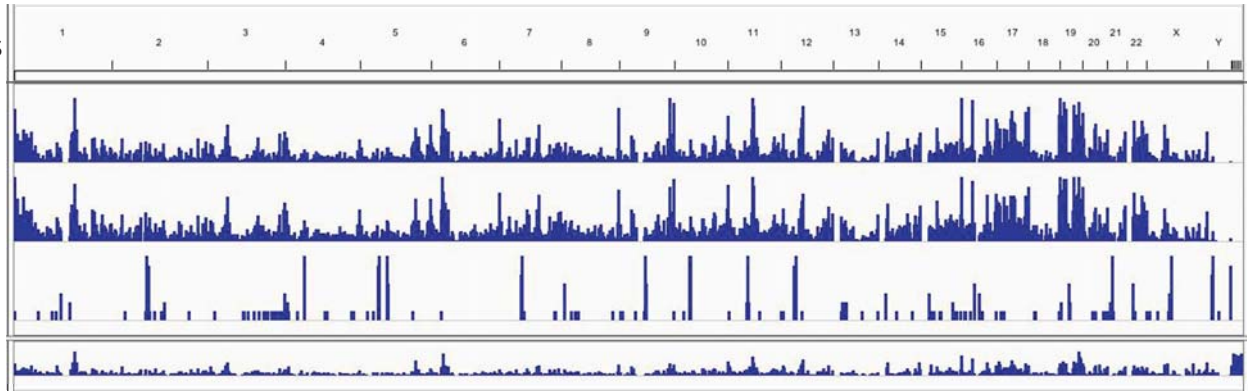


B

Chromosomes

H3K4me3
DHTH3K4me3
EtOHpY88-H4
IGF

Ref Genes



C

Rank Motif	p value	log P- p value	% of Targets	% of Backgrou nd	STD(Bg STD)	Best Match/Details
1	1.00E-251	-5.80E+02	58.51%	2.32%	2795.0bp (654.9bp)	PB0178.1_Sox8_2/Jaspar(0.677)
2	1.00E-228	-5.27E+02	35.11%	0.30%	2877.5bp (815.4bp)	MA0136.1_ELF5/Jaspar(0.609)
3	1.00E-220	-5.08E+02	33.24%	0.26%	3153.9bp (788.0bp)	STAT5(Stat)/mCD4+Stat5-ChIP-(GSE12346)/(0.873)
4	1.00E-214	-4.94E+02	35.90%	0.43%	2479.7bp (757.2bp)	PB0060.1_Smad3_1/Jaspar(0.631)
5	1.00E-211	-4.87E+02	35.90%	0.45%	3068.0bp (721.4bp)	MA0122.1_Nkx3-2/Jaspar(0.612)
6	1.00E-210	-4.85E+02	33.24%	0.32%	2739.3bp (943.7bp)	PB0166.1_Sox12_2/Jaspar(0.703)
7	1.00E-203	-4.68E+02	34.84%	0.45%	3096.8bp (714.0bp)	MA0052.2_MEF2A/Jaspar(0.667)
8	1.00E-197	-4.55E+02	35.64%	0.56%	3049.0bp (763.8bp)	MA0479.1_FOXH1/Jaspar(0.683)
9	1.00E-182	-4.19E+02	36.44%	0.81%	3087.0bp (719.7bp)	Tcf3(HMG)/mES-Tcf3-ChIP-(GSE11724)/Homer(0.657)
10	1.00E-177	-4.09E+02	28.46%	0.28%	2685.1bp (715.3bp)	CHR(?)Hela-CellCycle-Expression/Homer(0.755)
11	1.00E-169	-3.90E+02	28.19%	0.32%	2759.9bp (802.3bp)	PB0137.1_Irf3_2/Jaspar(0.733)
12	1.00E-165	-3.80E+02	36.44%	1.08%	3259.6bp (668.4bp)	MA0151.1_ARID3A/Jaspar(0.627)
13	1.00E-134	-3.10E+02	30.32%	0.92%	3190.7bp (595.7bp)	PB0111.1_Bhlhb2_2/Jaspar(0.583)
14	1.00E-131	-3.03E+02	22.34%	0.27%	2994.2bp (1016.1bp)	PB0137.1_Irf3_2/Jaspar(0.606)
15	1.00E-129	-2.98E+02	31.38%	1.16%	2836.1bp (665.4bp)	HRE(HSF)/HepG2-HSF1-ChIP-(GSE31477)/Homer(0.670)

D

Rank Motif	Name	p value	log P- p value	q value (Benja mini)	# Target Seque nces with Motif	% of Targets Sequenc es with Motif	# Backgrou nd Sequenc es with Motif	% of Backgrou nd Sequenc es with Motif
1	STAT5(Stat)/mCD4+Stat5-ChIP-(GSE12346)	1.00E-12	-2.96E+01	0	151	40.16%	9023.3	23.12%
2	STAT6(Stat)/Macrophage-Stat6-ChIP-(GSE38377)	1.00E-07	-1.78E+01	0	181	48.14%	13349.2	34.21%
3	CEBP-CEBP(bZIP)/MEF-Chop-ChIP-(GSE35681)	1.00E-05	-1.17E+01	0.0008	90	23.94%	5981.6	15.33%
4	ZFX(Zf)/mES-Zfx-ChIP-Seq-(GSE11431)	1.00E-02	-6.21E+00	0.1332	143	38.03%	12072.6	30.94%
5	Rfx2(HTH)/LoVo-RFX2-ChIP-Seq-(GSE49402)	1.00E-02	-4.74E+00	0.4621	16	4.26%	844.6	2.16%

Figure S4 (related to Figures 5 and 6). Genome wide deposition of the pY88-H4 epigenetic marks in PC and de novo motif enrichment

(A) LNCaP (Left), VCaP (middle) and LAPC4 (right) cells co-transfected with GFP and either FLAG-tagged AR construct or vector. Cells were treated with vehicle or (*R*)-9bMS (3 μ M) in androgen-deprived condition for 96 hr, followed by immunoblotting with respective antibody.

(B) ChIP-sequencing peak distribution of H4K4me3 and pY88-H4 marks in C4-2B cells treated with DHT, EtOH or IGF.

(C) ChIP-sequencing peaks were searched for enriched sequence motifs and the de novo motif enrichment is shown.

(D) ChIP-sequencing peaks were searched for known motif enrichment.

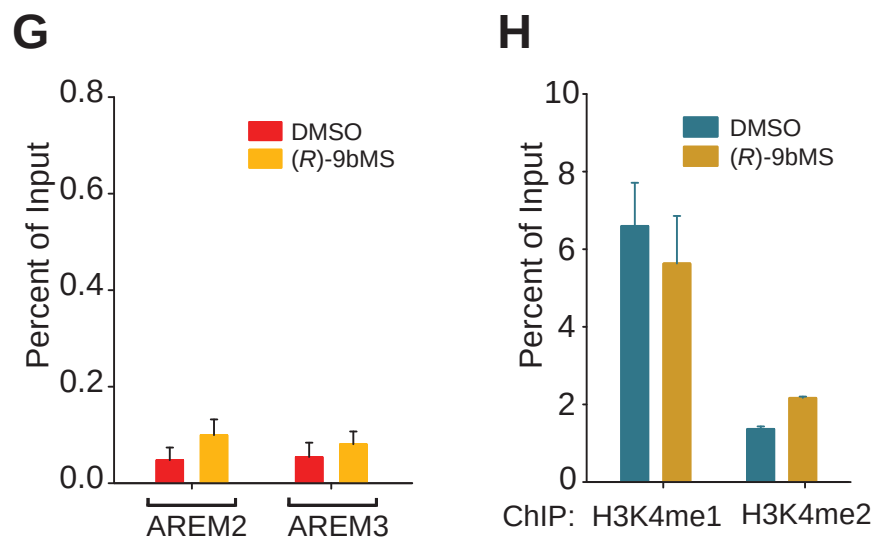
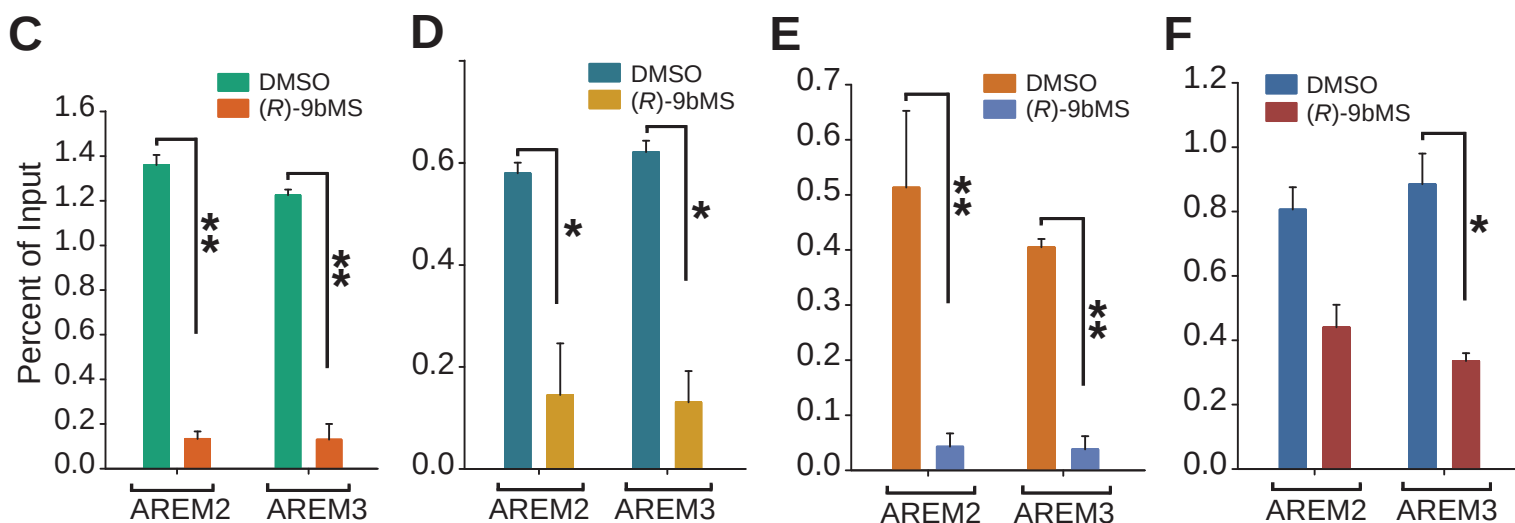
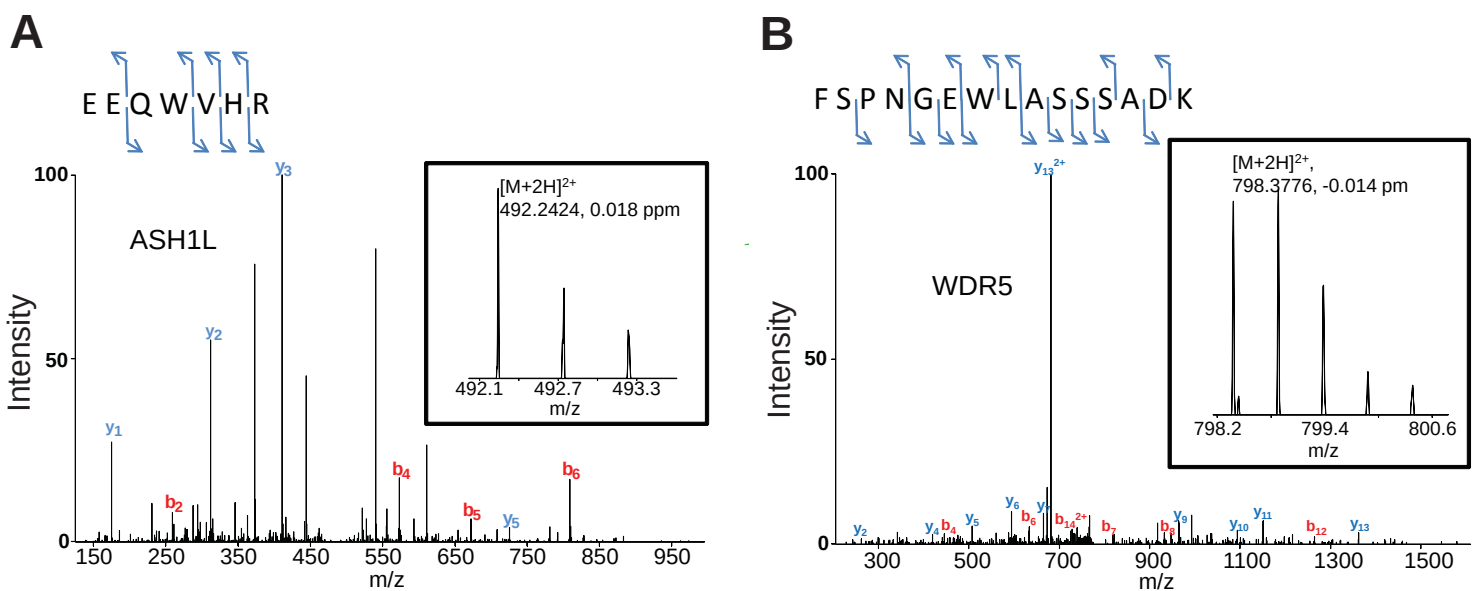


Figure S5 (related to Figure 6). Identification of ASH1L and WDR5 as pY88-H4 binding proteins

(A) ASH1L identified by LC-MS/MS. The doubly charged peptide was detected with mass-to-charge ratio 492.2424, which represents mass error of 0.018 ppm. The tandem mass spectrum matched the following sequence, EEQWVHR. The assignment was made with MaxQuant with a score of 116.5.

(B) WDR5 identified by LC-MS/MS. The doubly charged peptide was detected with mass-to-charge ratio 798.3776, which represents mass error of -0.014 ppm. The tandem mass spectrum matched the following sequence, FSPNGEWLASSSADK. The assignment was made with MaxQuant with a score of 66.3.

(C-G) Chromatin was isolated from C4-2B cells treated with vehicle or (*R*)-9bMS and ChIP was performed with WDR5 (C), MLL2 (D), H3K4me3 (E), RNA Pol II (F) and IgG (G) antibodies, followed by qPCR using primers corresponding to AREM2 or AREM3 (n = 2, three replicates).

(H) Chromatin was isolated from C4-2B cells treated with vehicle or (*R*)-9bMS and ChIP was performed using either anti-H3K4me1 or 2 antibody, followed by qPCR for AREM1 (n = 2, three replicates).

Data are represented as mean $\pm$ SEM as in (C-H). **p<0.01, *p<0.05.

Table S4. pY88-H4-interacting proteins identified using proteomics approach

Protein Descriptions	PEP
O14686 MLL2_HUMAN Histone-lysine N-methyltransferase MLL2	0.043214
Q9NR48 ASH1L_HUMAN Histone-lysine N-methyltransferase ASH1L	0.042511
P56180 TPTE_HUMAN Putative tyrosine-protein phosphatase TPTE	0.038838
P50053 KHK_HUMAN Ketohexokinase	0.026829
Q9HAN9 NMNA1_HUMAN Nicotinamide mononucleotide adenylyltransferase 1	0.022971
Q8NEY1 NAV1_HUMAN Neuron navigator 1	0.021591
Q96A99 PTX4_HUMAN Pentraxin-4	0.019817
O14678 ABCD4_HUMAN ATP-binding cassette sub-family D member 4	0.01971
Q8IUF8 MINA_HUMAN Bifunctional lysine-specific demethylase and histidyl-hydroxylase MINA	0.017435
Q8WXX5 DNJC9_HUMAN DnaJ homolog subfamily C member 9	0.0096056
O43347 MSI1H_HUMAN RNA-binding protein Musashi homolog 1	0.0094311
O75955 FLOT1_HUMAN Flotillin-1	0.0085269
Q8TAE8 G45IP_HUMAN Growth arrest and DNA damage-inducible proteins-interacting protein	0.007875
Q86VM9 ZCH18_HUMAN Zinc finger CCCH domain-containing protein 18	0.0057989
Q9UC06 ZNF70_HUMAN Zinc finger protein 70	0.0046104
Q15393 SF3B3_HUMAN Splicing factor 3B subunit 3	0.0041058
Q9P275 UBP36_HUMAN Ubiquitin carboxyl-terminal hydrolase 36	0.0024712
P10599 THIO_HUMAN Thioredoxin	0.0023493
P09234 RU1C_HUMAN U1 small nuclear ribonucleoprotein C	0.0023197
Q5T280 C1114_HUMAN Uncharacterized protein C9orf114	0.0022074
Q9NRW3 ABC3C_HUMAN DNA dC->dU-editing enzyme APOBEC-3C	0.0022012
Q9UHA3 RLP24_HUMAN Probable ribosome biogenesis protein RLP24	0.0019719
Q8IUX4 ABC3F_HUMAN DNA dC->dU-editing enzyme APOBEC-3F	0.0013902
P00492 HPRT_HUMAN Hypoxanthine-guanine phosphoribosyltransferase	0.0013154
Q9BZJ0 CRNL1_HUMAN Crooked neck-like protein 1	0.00092754
Q9H7E9 CH033_HUMAN UPF0488 protein C8orf33	0.00038028
Q14690 RRP5_HUMAN Protein RRP5 homolog	0.00034819
P49759 CLK1_HUMAN Dual specificity protein kinase CLK1	0.00030504
Q9Y5B9 SP16H_HUMAN FACT complex subunit SPT16	0.00024604
Q15633 TRBP2_HUMAN RISC-loading complex subunit TARBP2	8.14E-05
Q13573 SNW1_HUMAN SNW domain-containing protein 1	5.29E-05
Q9Y3F4 STRAP_HUMAN Serine-threonine kinase receptor-associated protein	3.08E-05
P14174 MIF_HUMAN Macrophage migration inhibitory factor	2.58E-05
P46109 CRKL_HUMAN Crk-like protein	2.51E-05
Q7Z7K6 CENPV_HUMAN Centromere protein V	1.76E-05
P84243 H33_HUMAN Histone H3.3	7.20E-06
P61964 WDR5_HUMAN WD repeat-containing protein 5	2.41E-06
Q08945 SSRP1_HUMAN FACT complex subunit SSRP1	3.23E-07
Q9H6Y2 WDR55_HUMAN WD repeat-containing protein 55	2.08E-07
Q15061 WDR43_HUMAN WD repeat-containing protein 43	6.33E-34
Q9UMS4 PRP19_HUMAN Pre-mRNA-processing factor 19	7.21E-35
Q9GZL7 WDR12_HUMAN Ribosome biogenesis protein WDR12	3.03E-53
O15213 WDR46_HUMAN WD repeat-containing protein 46	1.51E-60
Q8IWA0 WDR75_HUMAN WD repeat-containing protein 75	6.80E-81
Q9UNX4 WDR3_HUMAN WD repeat-containing protein 3	2.47E-102

Q9BZE4 NOG1_HUMAN Nucleolar GTP-binding protein 1	9.42E-104
Q6RFH5 WDR74_HUMAN WD repeat-containing protein 74	6.08E-105
Q8NI36 WDR36_HUMAN WD repeat-containing protein 36	5.73E-131

Table S5 (related to Figure 6). AR staining intensity versus BPH, PIN and prostate cancer disease stages and all pairwise comparisons by Tukey-Kramer Method

Group	Mean	SD	Min	Median	Max	p value
CRPC	7.5	0.7	6	8	8	<.0001*
BPH	1.7	2.7	0	0	7	
G6	5.0	1.7	0	6	7	
G7	6.5	1.5	0	7	8	
G8,9,10	6.3	1.8	0	7	8	
PIN	4.6	2.2	0	5	7	

* indicates the p value by F-test

Adjusted p value by the Tukey-Cramer Method					
	BPH	G6	G7	G8,9,10	PIN
CRPC	<.0001	0.0001	0.41	0.18	<.0001
BPH		<.0001	<.0001	<.0001	<.0001
G6			0.001	0.024	0.97
G7				0.96	<.0001
G8,9,10					0.001

Table S6 (related to Figure 6). H3K4me3 staining intensity versus BPH, PINs and prostate cancer disease stages and all pairwise comparisons by Tukey-Kramer Method

Group	Mean	SD	Min	Median	Max	p value
CRPC	7.6	0.6	6	8	8	<.0001 [*]
BPH	5.9	1	3	6	7	
G6	6.5	0.9	5	7	8	
G7	6.8	0.8	5	7	8	
G8,9,10	7.0	1	3	7	8	
PIN	6.6	0.9	4	7	8	

* indicates the p value by F-test

Adjusted p value by Tukey-Cramer Method					
	BPH	G6	G7	G8,9,10	PIN
CRPC	<.0001	0.001	0.014	0.15	0.001
BPH		0.15	0.0003	<.0001	0.06
G6			0.48	0.10	>0.99
G7				0.85	0.72
G8,9,10					0.20

Table S7 (related to Figure 6). Pearson’s correlation coefficients between pY88-H4, H3K4me3, and AR expression

	AR	H3K4me3
pY88-H4	0.30 (p <0.0001)	0.23 (p=0.001)
AR		0.66 (p <0.0001)

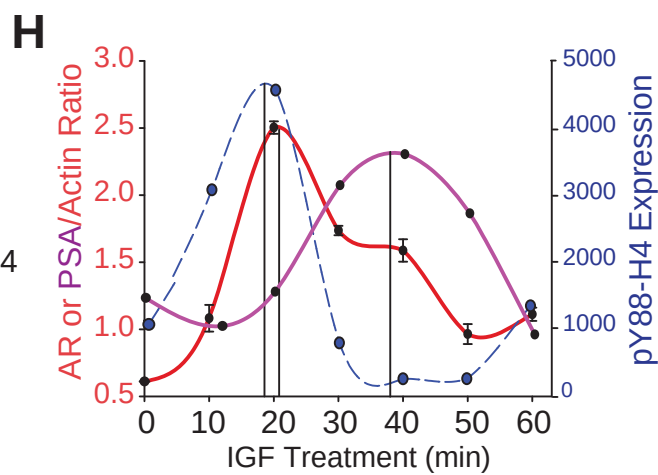
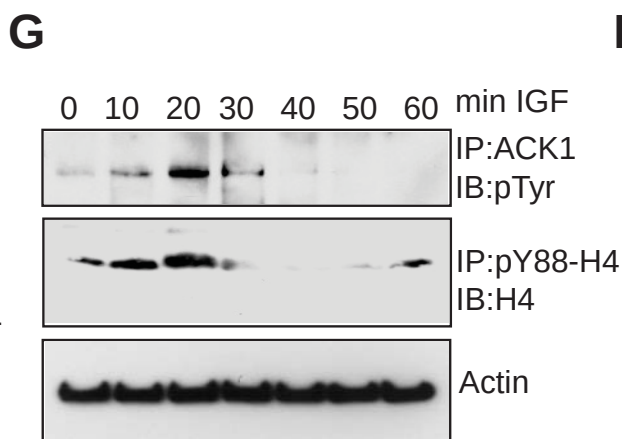
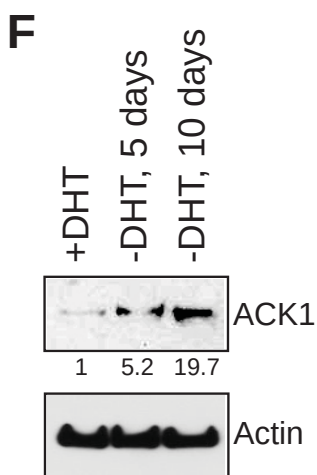
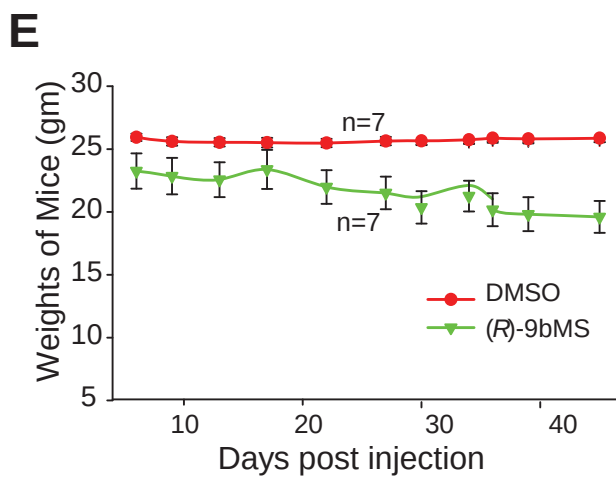
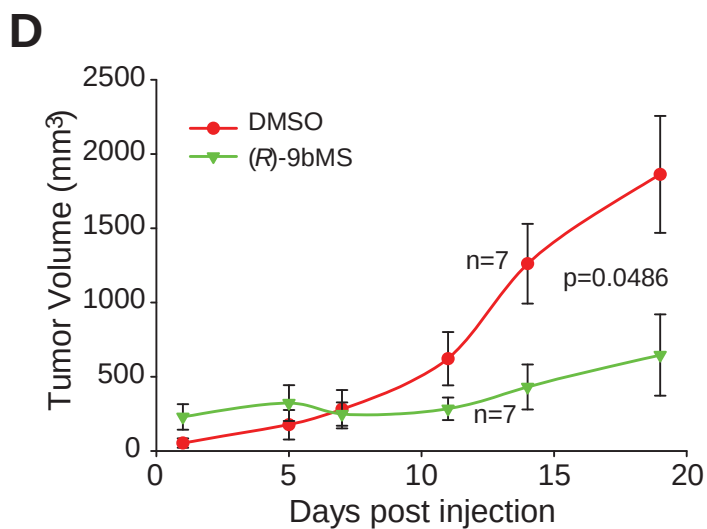
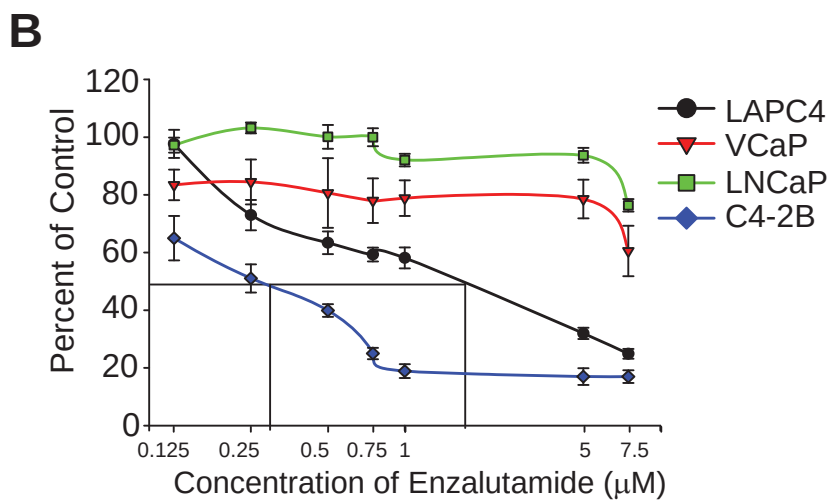
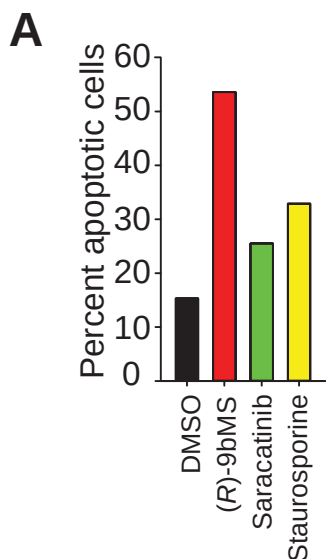


Figure S6 (related to Figure 7). ACK1 inhibition causes suppression of CRPC xenograft tumor growth

(A) VCaP cells treated with vehicle or (*R*)-9bMS, saracatinib or staurosporine, stained with Caspase-3/7 detection agent and SYTOX® Dead cell stain and number of apoptotic cells was counted by flow cytometry.

(B) PC cells treated with indicated concentrations of Enzalutamide for 96 hr and viable cells were counted by trypan blue dye exclusion assay (*n* = 3, three replicates).

(C) C4-2B xenograft tumors were excised from mice treated with vehicle or (*R*)-9bMS, the representative tumors are shown (left panel). A tumor that was metastasized to prostate gland (tumor weight = 1.2 gm) in vehicle treated mice is shown (middle panel). A tumor that was detected to be attached to rib case in (*R*)-9bMS treated mice (tumor weight = 0.4 gm) is shown on right.

(D) Male nude mice were castrated and injected subcutaneously with LNCaP-caAck cells. When tumors became palpable, the mice were injected with either vehicle (10% DMSO in PBS) or (*R*)-9bMS (50 mg/kg of body weight) for 6 days a week for 4 weeks (*n* = 7 mice for each treatment). Tumor volumes were measured using calipers.

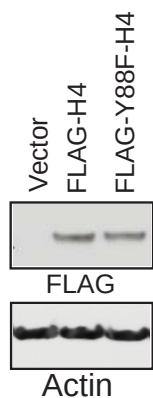
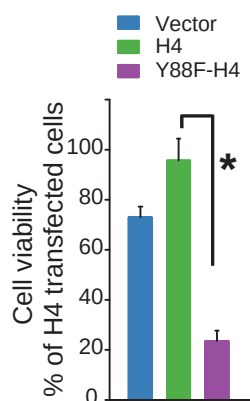
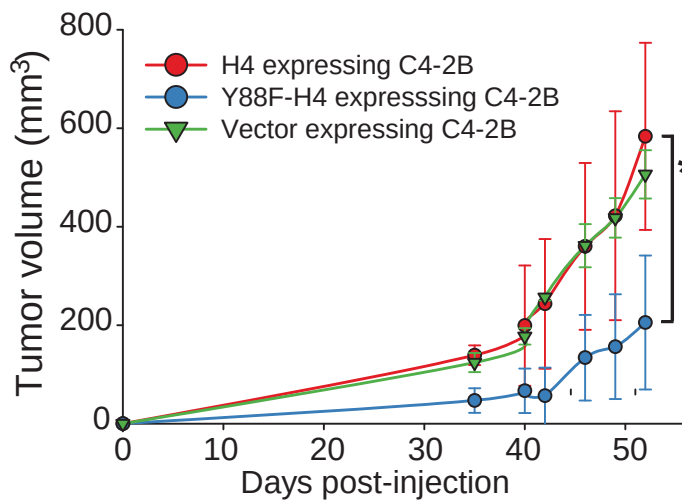
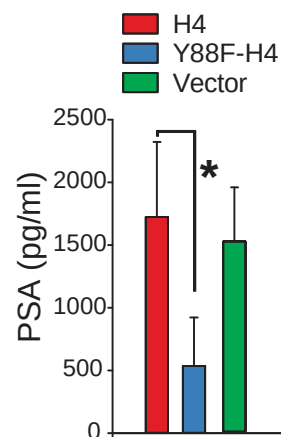
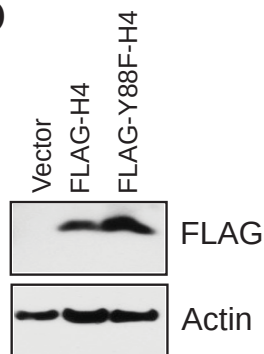
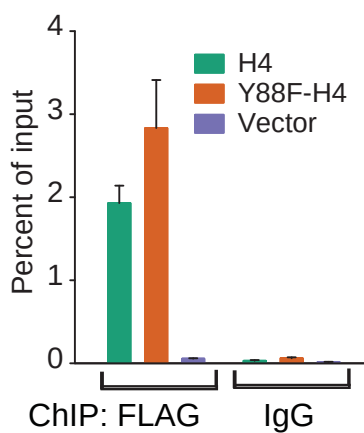
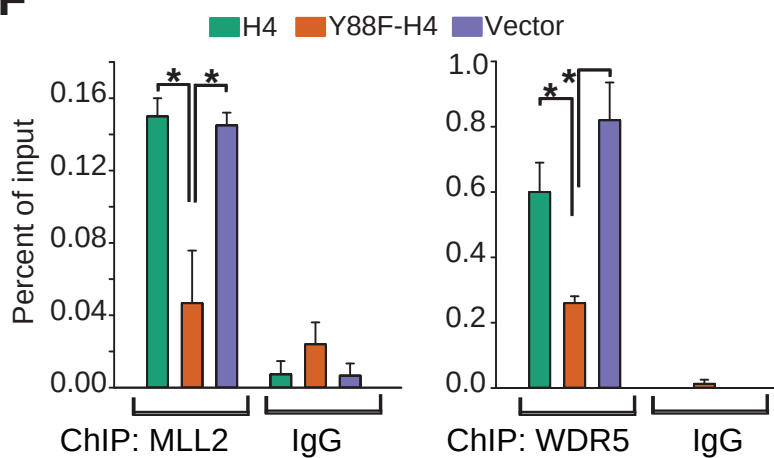
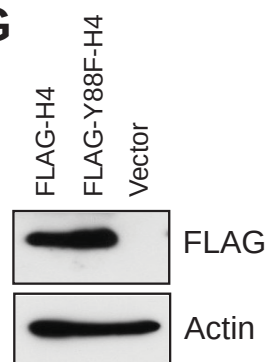
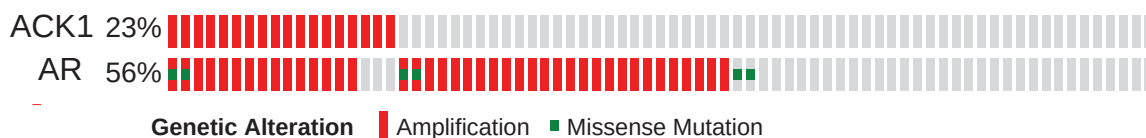
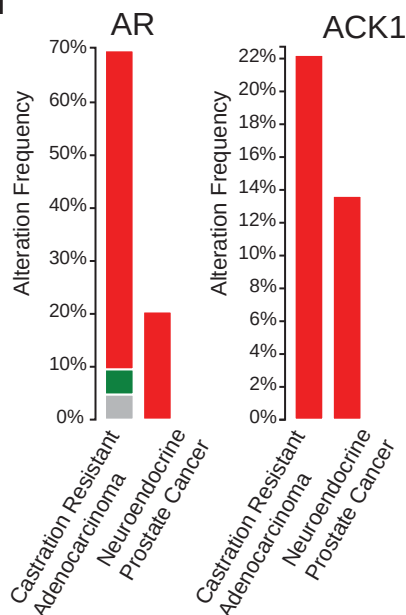
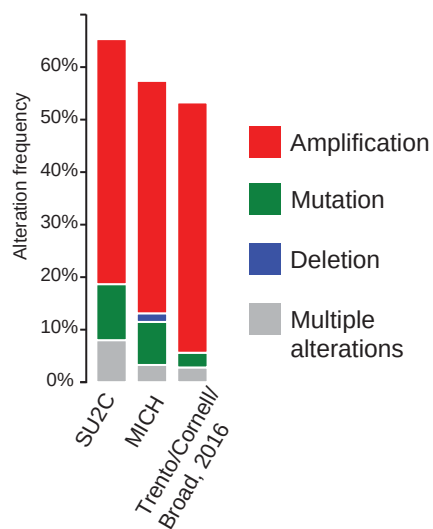
(E) Weights of the SCID mice injected with vehicle or (*R*)-9bMS, shown in Figure 7F.

(F) LAPC4 cells were either treated with DHT (100 nM) or deprived of androgen for 5 to 10 days and equal amounts of cell lysates were immunoblotted.

(G) Growth factor-deprived C4-2B cells were treated with IGF for 10 to 60 min. Lysates were immunoprecipitated with ACK1 antibody followed by immunoblotting with pTyr antibody (top). Lysates were also immunoprecipitated with pY88-H4 antibody followed by immunoblotting with H4 antibody (middle panel). Lower panel is immunoblotted for actin.

(H) RNA prepared from growth factor-deprived C4-2B cells were treated with IGF followed by qRT-PCR with AR, PSA or actin primers. The data is presented along with quantitation of pY88-H4 expression, shown in Figure S6G.

Data are represented as mean $\pm$ SEM as in (B, D and E).

A**B****C****D****E****F****G****H****I****J****K**

Gene Set is altered in 57 (53.3%) of queried samples		
☒ Mutual exclusivity	☒ Co-occurrence	☐ Significant pairs
Gene A	Gene B	p value
ACK1	AR	0.002
Log Odds Ratio	Association	
1.687	Tendency towards co-occurrence	
	Significant	

Figure S7 (related to Figure 7). The Y88F mutant of H4 expressing C4-2B cells are compromised in CRPC growth

(A) LNCaP cells electroporated with ACK1 and FLAG-tagged H4 or Y88F-H4 mutant constructs were grown in androgen-starved condition for 6 days, followed by counting of viable cells (n = 2, three replicates).

(B) C4-2B cells expressing FLAG-tagged H4 or Y88F mutant of H4 or vector were implanted subcutaneously in castrated male SCID mice and grown until tumors reached ~ 600 mm³.

(C) PSA levels in serum obtained from the mice (Figure S7B) were determined by ELISA.

(D) Tumors were excised and the xenograft tumor lysates were immunoblotted for FLAG or actin.

(E) C4-2B cells transfected with FLAG-tagged H4 or Y88F mutant of H4 or vector followed by ChIP with anti- FLAG or IgG antibodies followed by qPCR for AREM2 (n = 2, three replicates).

(F) C4-2B cells were transfected with FLAG-tagged H4 or Y88F mutant of H4 or vector and ChIP was performed with MLL2, WDR5 and IgG antibodies followed by qPCR for AREM2 (n = 2, three replicates).

(G) C4-2B cells transfected with either FLAG-tagged H4 or Y88F mutant of H4 or vector (as shown in S7E and S7F) and immunoblotted for FLAG and actin.

(H) Alterations in AR and ACK1 gene set pathway were assessed in human CRPCs (TCGA dataset; cBioPortal).

(I) Gene amplification of AR & ACK1 was analyzed in CRPCs (cBioPortal).

(J) Alterations in AR and ACK1 assessed in CRPCs (cBioPortal).

(K) Co-occurrence analysis of AR and ACK1 in CRPCs (cBioPortal).

Data are represented as mean ± SEM as in (A-C, E and F). **p<0.01, *p<0.05.

WEE1 epigenetically modulates 5-hmC levels by pY37-H2B dependent regulation of *IDH2* gene expression

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ABSTRACT

Epigenetic signaling networks dynamically regulate gene expression to maintain cellular homeostasis. Previously, we uncovered that WEE1 phosphorylates histone H2B at tyrosine 37 (pY37-H2B) to negatively regulate global histone transcriptional output. Although pY37-H2B is readily detected in cancer cells, its functional role in pathogenesis is not known. Herein, we show that WEE1 deposits the pY37-H2B marks within the tumor suppressor gene, isocitrate dehydrogenase 2 (*IDH2*), to repress transcription in multiple cancer cells, including glioblastoma multiforme (GBMs), melanoma and prostate cancer. Consistently, GBMs and primary melanoma tumors that display elevated WEE1 mRNA expression exhibit significant down regulation of the *IDH2* gene transcription. *IDH2* catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), an essential cofactor for the TET family of 5-methylcytosine (5mC) hydroxylases that convert 5-mC to 5-hydroxymethylcytosine (5-hmC). Significantly, the WEE1 inhibitor AZD1775 not only abrogated the suppressive H2B Y37-phosphorylation and upregulated *IDH2* mRNA levels but also effectively reversed the 'loss of 5-hmC' phenotype in melanomas, GBMs and prostate cancer cells, as well as melanoma xenograft tumors. These data indicate that the epigenetic repression of *IDH2* by WEE1/pY37-H2B circuit may be a hitherto unknown mechanism of global 5-hmC loss observed in human malignancies.

INTRODUCTION

The precise execution of epigenetic signaling events, which includes both addition and removal of epigenetic marks in a temporally regulated manner, is vital to preserve the chromatin landscape and maintain cellular homeostasis. Deregulated epigenetic signaling networks profoundly alter the epigenome that is linked to cancer

and developmental diseases [1–6]. It has become evident that behind every histone or DNA modification, commonly referred to as epigenetic changes, there are a number of regulators that work in a highly orchestrated manner to govern the transcriptional landscape and determine cell fate. Modification of tyrosine residues in proteins functions as a precise on-off switch and has acquired considerable significance particularly in light of the evidence of histones

being modified directly by disparate kinases in recent years [7–14]. Of the 6 known histone tyrosine phosphorylation events, WEE1 directly phosphorylates the histone H2B at tyrosine 37 (pY37-H2B) to negatively regulate global histone transcriptional output in a temporally regulated manner during each cell cycle [7, 13]. The histone transcriptional repressor, HIRA ‘a reader’ of the pY37-H2B epigenetic marks, recognizes these marks, displaces a transcriptional coactivator to suppress global histone mRNA synthesis at the end of DNA synthesis. Interestingly, WEE1 kinase is reported to be aberrantly expressed in malignant melanomas, GBMs and triple-negative and luminal breast cancers [15–19]. The WEE1 inhibitor, AZD1775, also known as MK-1775 [20, 21] potently radiosensitizes human tumor cells [22, 23], suggesting that these tumors rely on WEE1 kinase activity for chromatin integrity. To better understand how cancer cells may benefit from this newly discovered WEE1 epigenetic activity, we mined our pY37-H2B chromatin immunoprecipitation (ChIP)-sequencing data for genes associated with oncogenesis; it revealed that pY37-H2B marks were deposited at an important tumor suppressor gene—the isocitrate dehydrogenase 2 (*IDH2*).

The isocitrate dehydrogenases, *IDH1* and *IDH2*, catalyze the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), a critical regulator of cell metabolism [24, 25]. About 60 dioxygenases expressed in mammalian cells utilize α -KG as an essential cofactor in the oxidation reaction, including the recently discovered TET family of 5-methylcytosine (5mC) hydroxylases that convert 5-mC to 5-hydroxymethylcytosine (5-hmC). A significant decrease or loss of 5-hmC has been identified as a recurrent epigenetic phenomenon in a large number of human malignancies, including melanomas, glioblastoma multiforme (GBM), clear cell renal cell carcinoma, urothelial cell carcinoma, breast, liver, lung, pancreatic and prostate cancers [26–30]. The significance of 5-hmC loss specifically in cancer cells became further evident when reintroduction of the 5-hmC by recombinant *IDH2* or TET2, suppressed tumor growth and increased tumor-free survival in the melanoma mouse models [26]. Taken together, it has become apparent that the global loss of 5-hmC is a significant occurrence characteristic of malignancy [31, 32], opening the selective restoration of 5-hmC levels as a new therapeutic option with small molecule inhibitors.

While the down-regulation of *IDH2* is linked to the loss of 5-hmC in human tumors, the epigenetic mechanisms by which *IDH2* gene expression is repressed in human tumors is not known. Moreover although mutations in the *IDH1/IDH2* genes underlie global 5hmC loss [24], these do not account for all the cases observed [27, 32]. Serendipitously, we uncovered that deposition of pY37-H2B epigenetic marks in the *IDH2* gene directly correlated with reduced gene expression. Further, this decrease in *IDH2* gene expression correlated with reduced 5-hmC levels, suggesting that the overexpression of WEE1 in cancer cells may suppress the expression of *IDH2*, leading to decreased

5-hmC levels. Further, increased WEE1 expression in glioblastomas and melanomas positively correlated with decreased *IDH2* expression levels. Conversely, inhibition of WEE1 kinase activity by the selective inhibitor AZD1775, erased the pY37-H2B repressive marks leading to a marked increase in the levels of *IDH2*, thus reversing the ‘loss of 5-hmC’ phenotype. Overall, our data reveal a novel mode of WEE1 mediated *IDH2* downregulation as a recurrent epigenetic alteration that may be operational in melanomas, GBMs and prostate cancers.

RESULTS

H2B Y37-phosphorylation is a hallmark of multiple cancer cell lines

To interrogate a role for WEE/pY37-H2B epigenetic regulation in promoting human malignancies, melanoma (WM1366), prostate (LNCaP and LAPC4) and brain (T98G) cancer cell lines were first examined. H2B Y37-phosphorylation was a significant feature in several cancer cell lines tested (Figure 1A–1D, top panels). To determine whether pY37-H2B deposition can be reversed, cancer cells of multiple origins were treated with AZD1775, a WEE1 selective inhibitor. H2B Y37-phosphorylation was inhibited in all cancer models following treatment with AZD1775 (Figure 1A–1D, top panels).

To determine the consequence of suppression of H2B Y37-phosphorylation, various melanoma (SK-MEL-2, WM1363, MO5, IPC298 and WM1366), prostate (LNCaP-C4 and LAPC4), GBM (T98G) and HEK293 cells were treated with AZD1775 and the effect on cell proliferation was assessed (Figure 1E). The cells lines tested were highly sensitive to AZD1775-IC₅₀ values ranged from 100–500 nM (Figure 1F). An increased accumulation of cells in S phase was observed as a result of treatment with AZD1775 (Supplementary Figure 1). Combined, these data demonstrate that WEE1 mediated H2B Y37-phosphorylation is a recurrent pro-proliferative epigenetic modification in multiple proliferating cancer cells that can be specifically targeted with the WEE1 inhibitor, AZD1775.

WEE1 marks the *IDH2* gene locus with H2B Y37-phosphorylation

Earlier, we demonstrated that the WEE1 epigenetic activity is temporally regulated wherein the pY37-H2B epigenetic marks are specifically deposited at the end of S phase during each cell cycle [7]. We interrogated how H2B Y37-phosphorylation influences the transcriptional landscape to regulate cancer cell survival. Towards this goal, the genomic distribution of pY37-H2B epigenetic marks in normal cells, mouse embryo fibroblasts (MEFs) were examined. To identify the genes that are specifically regulated by WEE1, MEFs were synchronized by double

thymidine and were harvested 6.30 hours post-release in fresh media. Based on the cell cycle analysis that we have performed earlier, the 6.30 hours post-release time point represents the late S phase, prior to entry into G2/M phase of cell cycle [7]. ChIP-sequencing was performed using pY37-H2B antibodies as described earlier [7]. It revealed that pY37-H2B marks are

deposited not only upstream of *Hist1* locus, where large number of histone coding genes are located, but also in many other genes [7]. We performed detailed analysis of the pY37-H2B ChIP-sequencing data and uncovered that these epigenetic marks are deposited in the mouse *Idh2* intron1-2 (80,114,198 to 80,114,864 nt) (Figure 2A and Supplementary Table 1).

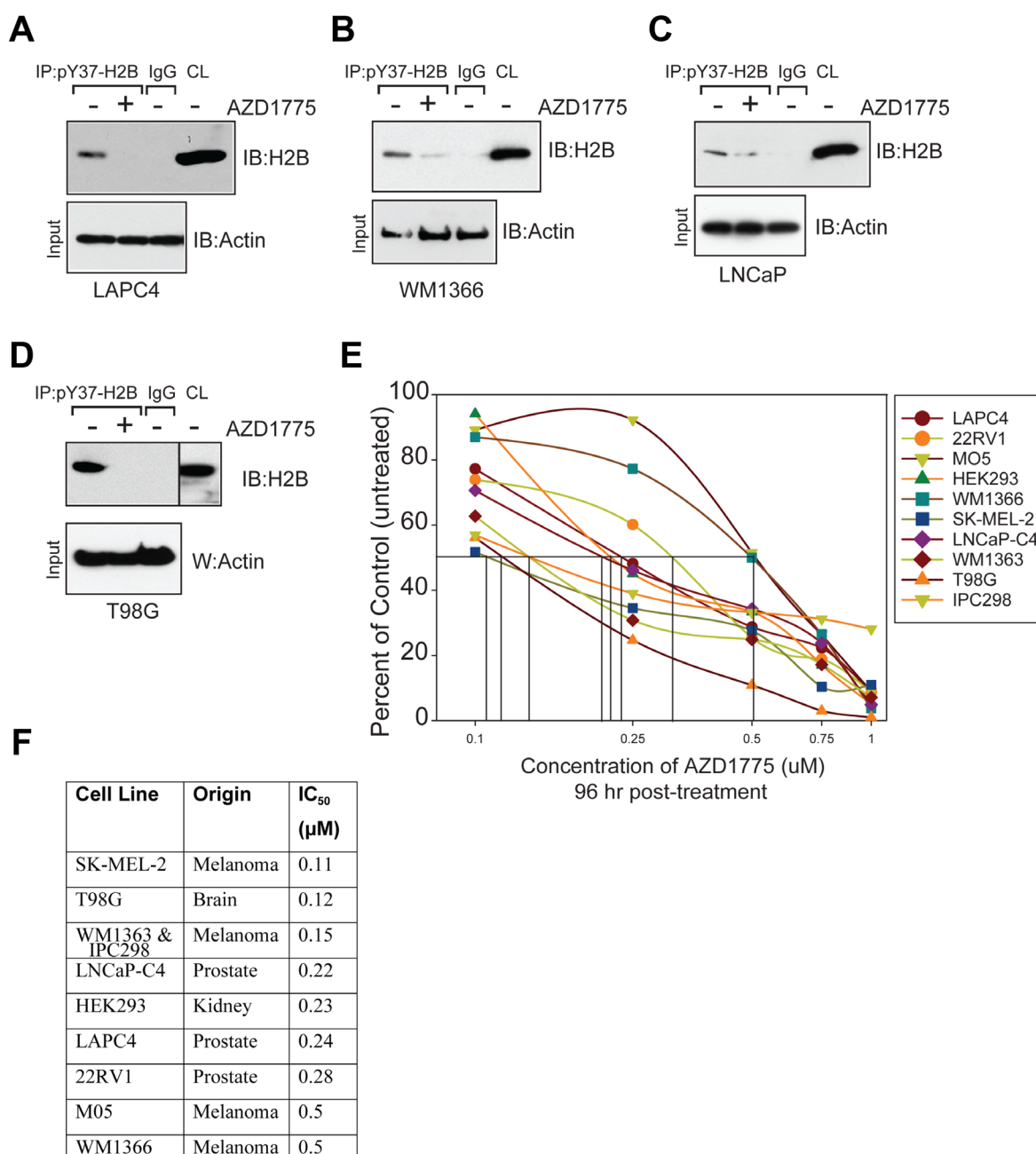


Figure 1: WEE1 inhibition suppresses pY37-H2B expression. (A–D) Prostate cancer (LAPC4 and LNCaP), melanoma (WM1366) and brain cancer (T98G) derived cells were treated with AZD1775 (2 μM, 24 hr) and lysates were immunoprecipitated with pY37-H2B antibodies, followed by immunoblotting with pan-H2B antibodies (top panels). CL indicates the crude lysates. Lower panel indicates immunoblotting of lysates with actin antibodies. (E) Cell viability assay. Melanoma, prostate and brain cancer cell lines were treated with AZD1775 for 96 hr and live/dead population was assessed by Trypan blue staining. Data represent mean ± standard error of the mean or s.e.m. ($n = 3$). (F) Table indicates IC₅₀ values based on cell proliferation analysis in AZD1775 treated cells in comparison to untreated cells shown in Figure 1E.

To validate the deposition of the pY37-H2B epigenetic marks within the mouse *Idh2* coding region, MEFs were synchronized by double thymidine and were harvested immediately (0 hours post-release) or 6.30 hours post-release in fresh media, which corresponds to G1/S and late S phase of cell cycle, respectively. Directed ChIP-qPCR was performed, which confirmed deposition within the *Idh2* gene at 6.30 hours but not at 0 hours, when WEE1 epigenetic activity is undetectable (Figure 2B). Further, treatment of cells with AZD1775 abolished the deposition of pY37-H2B epigenetic marks at *Idh2* locus. As a control, we used primers corresponding to the 'Gene desert' on chromosome 6, where pY37-H2B marks were not detected (control site, Figure 2C). To identify the corresponding region in human *IDH2* gene, we performed chromatin immunoprecipitation (ChIP) with pY37-H2B antibodies followed by PCR with 6 sets of primers spanning *IDH2* gene (90,083,978 to 90,102,554 nt). This led to the identification of the putative pY37-H2B-binding site in human cells (90,087,067 -90,087,236) located in exon 7 and part of intron 7 (Figure 2A and Supplementary Table 1). The primer sequences for human *IDH2* and mouse *Idh2* CHIP are shown in the methods section.

To examine the presence of pY37-H2B marks within the human *IDH2* gene locus, melanoma derived cell lines IPC298 and SK-MEL2, prostate cancer derived cell lines-LAPC4 and GBM derived cell line T98G were treated with WEE1 kinase inhibitor AZD1775 and sheared chromatin was prepared. ChIP was performed using the pY37-H2B antibody, followed by qPCR using primers corresponding to the pY37-H2B-binding site in the human *IDH2* gene. A significant decrease in the pY37-H2B deposition in the *IDH2* gene was observed upon WEE1 inhibition (Figure 2D–2G). To further validate a direct role for WEE1 in the deposition of the pY37-H2B epigenetic marks in human *IDH2*, ChIP was performed for the HEK293 cells that were transfected with the WEE1 kinase, followed by qPCR for pY37-H2B binding sites with the selected primer pairs. A significant increase in the pY37-H2B deposition in the *IDH2* gene was observed in WEE1 overexpressing cells (Figure 2H and 2I). Taken together these data indicate the ability of WEE1 to epigenetically mark *IDH2* locus is conserved in both mouse and human cells.

WEE1/pY37-H2B epigenetic axis suppresses *IDH2* gene transcription

To investigate whether WEE1/pY37-H2B signaling plays a direct role in regulating *IDH2* gene transcription, various melanoma (WM1963, IPC298, WM1396, WM3918, SK-MEL-2, WM38 and WM1366), prostate (LNCaP and LAPC4), GBM (U87) and HEK293 cells were treated with WEE1-specific inhibitor, AZD1775. RNA was prepared followed by real time PCR with *IDH2*-specific primers. A significant increase in *IDH2* transcript levels was observed upon WEE1 inhibition (Figure 3A–3K).

As a control, we utilized a primary human keratinocytes; interestingly, *IDH2* mRNA levels remained unchanged in these non-cancerous cells despite AZD1775 treatment (Figure 3L), suggesting a cancer specific regulation of *IDH2* by pY37-H2B/WEE1 signaling axis.

To examine whether increased *IDH2* mRNA levels upon AZD1775 treatment is not due to an 'off-target effect' of the inhibitor, LNCaP cells were transfected with WEE1-specific siRNA and control siRNA. A significant upregulation of *IDH2* mRNA levels was observed upon WEE1 knockdown in LNCaP cells (Figure 3M). Earlier we demonstrated that WEE1 negatively regulates global histone output [7]. Consistent with this notion, WEE1 inhibition resulted in a significant increase in histone H2A mRNA levels (Figure 3N). Taken collectively, these data indicate that the deposition of the pY37-H2B epigenetic marks by WEE1 has a repressive effect on *IDH2* gene regulation.

To assess whether the upregulation of *IDH2* gene transcription by WEE1 inhibition also reflects in *IDH2* protein levels, melanoma (B16) and prostate (LAPC4) cells were treated with AZD1775 and lysates were immunoblotted with *IDH2* antibodies. A distinct increase in the *IDH2* protein expression was observed upon WEE1 inhibition (Figure 4A). We also assessed whether WEE1 epigenetically regulates *IDH1* gene expression by performing qRT-PCR. AZD1775 treatment did not affect *IDH1* mRNA expression (Supplementary Figure 2).

Loss of *IDH2* function has previously been linked to decreased 5-hmC levels in human tumors [26–30]. Ease of transfection of WM1361, WM1366, LNCaP and IPC298 has made us use of these four cell lines to transfect with *IDH2* siRNA and detect 5hmC, as shown in Figure 4B–4E. To validate loss of *IDH2* lead to downregulation of 5-hmC levels, human cancer cells were transfected with *IDH2* siRNA and DNA was isolated followed by immunoblotting. A significant decrease in global 5-hmC levels was observed upon *IDH2* knockdown (Figure 4B). Conversely, overexpression of *IDH2* led to an increase in 5-hmC levels compared to vector control (Figure 4C).

We also assessed the effect of WEE1 loss on 5-hmC levels. siRNA mediated downregulation of WEE1 lead to a significant increase in 5-hmC levels, while overexpression of WEE1 resulted in a significant decrease in 5-hmC levels (Figure 4D and 4E). Taken together, these data suggests that WEE1/pY37-H2B signaling has an important role to play in regulating *IDH2* expression in multiple models.

5-hmC loss in cancer cells can be reversed by WEE1 inhibition

To determine whether WEE1 mediated *IDH2* transcriptional suppression also impacts 5-hmC levels in different cancer models, melanoma (SBC12, WM1366, WM38, SK-MEL-2, B16, IPC298 and MO5), GBM (T98G) and prostate cancer cells (LAPC4 and 22RV1) were treated with AZD1775 and total genomic DNA was

extracted to analyze the 5-hmC status. Genomic DNA was denatured and then blotted onto nitrocellulose membrane, followed by immunoblotting with the 5-hmC antibodies. A significant increase in 5-hmC levels occurred in AZD1775 treated cancer cell lines (Figure 5A). The blot

was also stained with methylene blue to confirm equal amounts of blotted DNAs (Figure 5A). The blots were quantitated which revealed that most cell lines exhibited about 2–9 fold increase in 5-hmC levels upon WEE1 inhibition (Figure 5B).

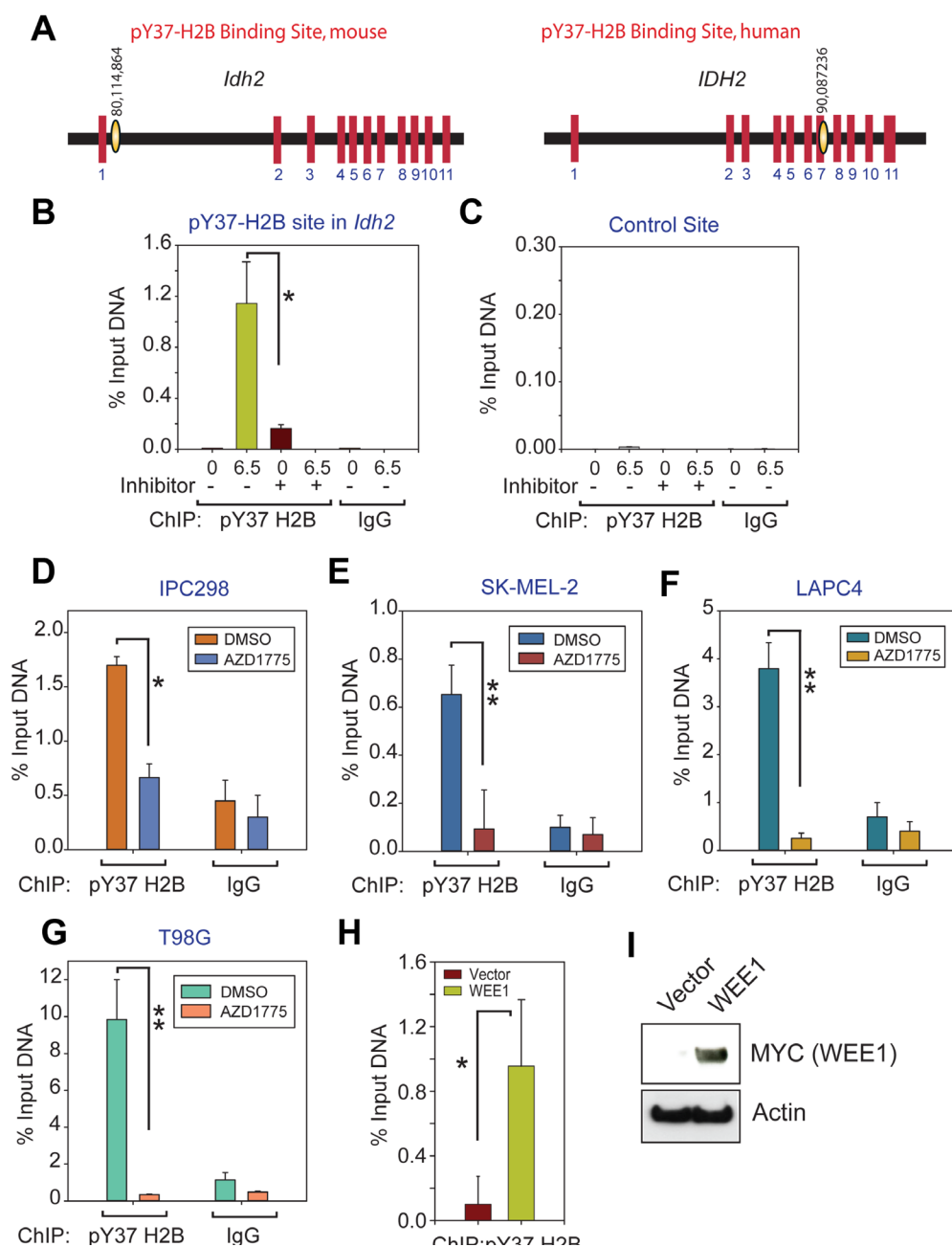


Figure 2: Deposition of WEE1 mediated pY37-H2B epigenetic marks within the *IDH2* gene locus. (A) Deposition of pY37-H2B epigenetic marks in the mouse *Idh2* and human *IDH2* locus are shown. The red boxes are the exons and yellow ovals are the sites of pY37-H2B deposition. (B) ChIP with pY37-H2B and IgG antibodies, followed by qPCR with primers corresponding to the pY37-H2B binding site of mouse embryo fibroblasts (MEFs). Data represent mean $\pm$ s.e.m. ($n = 3$, * $p < 0.01$). (C) ChIP with pY37-H2B and IgG antibodies, followed by qPCR with primers corresponding to the control site of MEFs untreated or treated with AZD1775. Data represent mean $\pm$ s.e.m. ($n = 3$). (D–G) ChIP with pY37-H2B and IgG antibodies, followed by qPCR with primers corresponding to the pY37-H2B binding site of human *IDH2* in IPC298, SK-MEL-2, LAPC4 and T98G cells. Data represent mean $\pm$ s.e.m. ($n = 3$). * $p < 0.05$, ** $p < 0.01$. (H) ChIP-qPCR of chromatin isolated from HEK293 cells transfected with the vector and WEE1 expressing constructs. Data represent mean $\pm$ s.e.m. ($n = 3$). * $p < 0.01$. (I) Immunoblotting of HEK293 lysates transfected with MYC-tagged WEE1 using anti-MYC antibodies.

To assess whether the global increase in 5-hmC levels upon WEE1 inhibition can be suppressed by forced *IDH2* knock-down, we transfected LNCaP cells with *IDH2* siRNA and compared with pharmacological blockade following treatment with AZD1775. A significant decrease in global 5-hmC levels was seen upon *IDH2* knock-down in LNCaP

cells that was not upregulated in response to AZD1775 treatment (Figure 5C). Taken together with the observed increase in *IDH2* expression, shown in Figure 3, these data indicate that WEE1 mediated deposition of pY37-H2B marks in *IDH2* gene results in transcriptional silencing, causing a significant decrease in global 5-hmC levels.

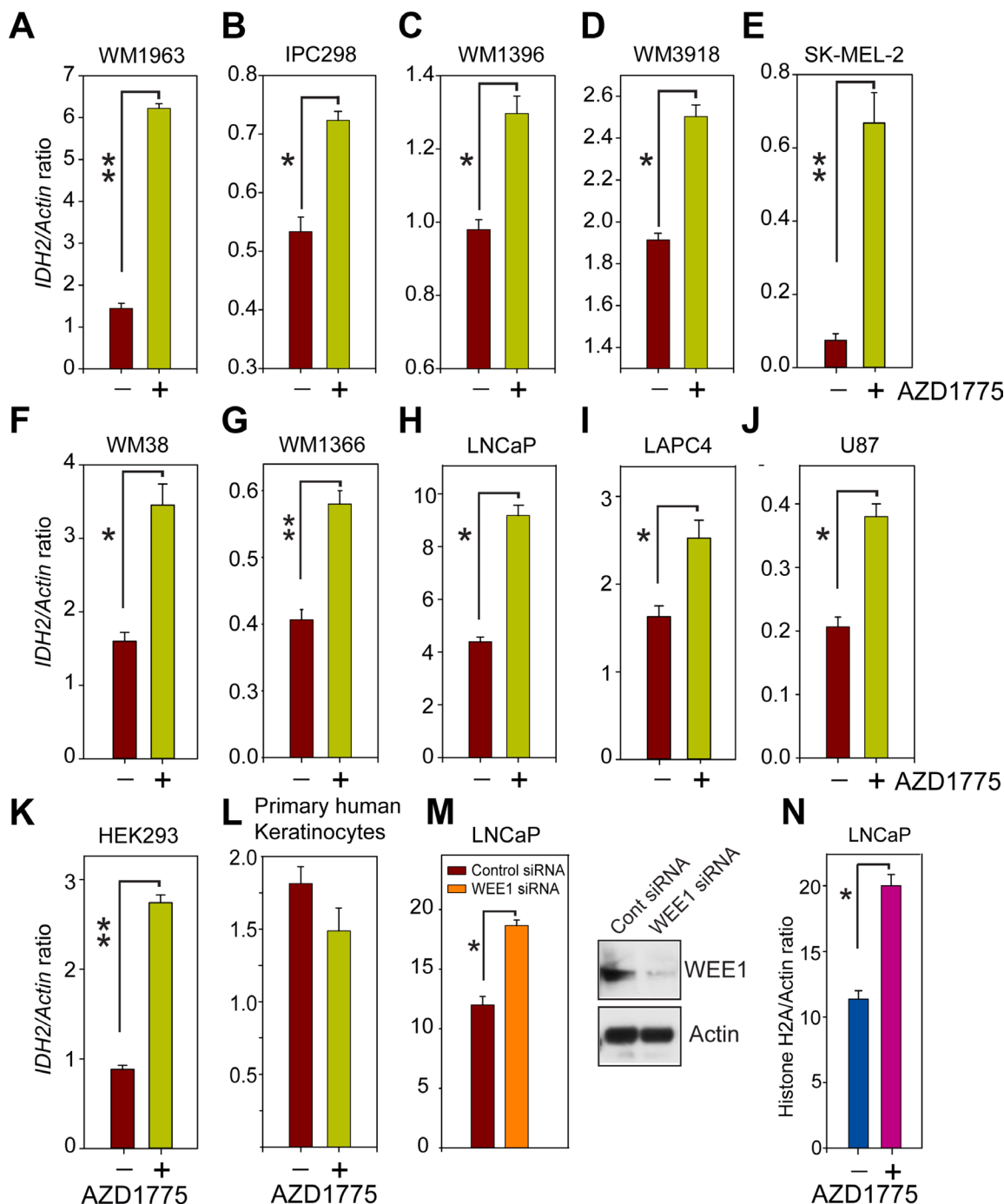


Figure 3: Inhibition of WEE1 kinase activity enhances *IDH2* mRNA expression. (A–L) Quantitative reverse transcription polymerase chain reaction (qRT–PCR) analysis of *IDH2* mRNA expression in indicated cells treated with AZD1775 (1 μ M, 24 hr). Data represent mean $\pm$ s.e.m. ($n = 3$). * $p < 0.05$, ** $p < 0.01$. (M) qRT–PCR analysis of *IDH2* in LNCaP cells transfected with WEE1 siRNA. Data represent mean $\pm$ s.e.m. ($n = 3$). * $p < 0.05$. Loss of WEE1 expression is confirmed by immunoblotting. (N) qRT–PCR analysis of histone H2A mRNA in LNCaP cells treated with WEE1 inhibitor, AZD1775. Data represent mean $\pm$ s.e.m. ($n = 3$). * $p < 0.05$.

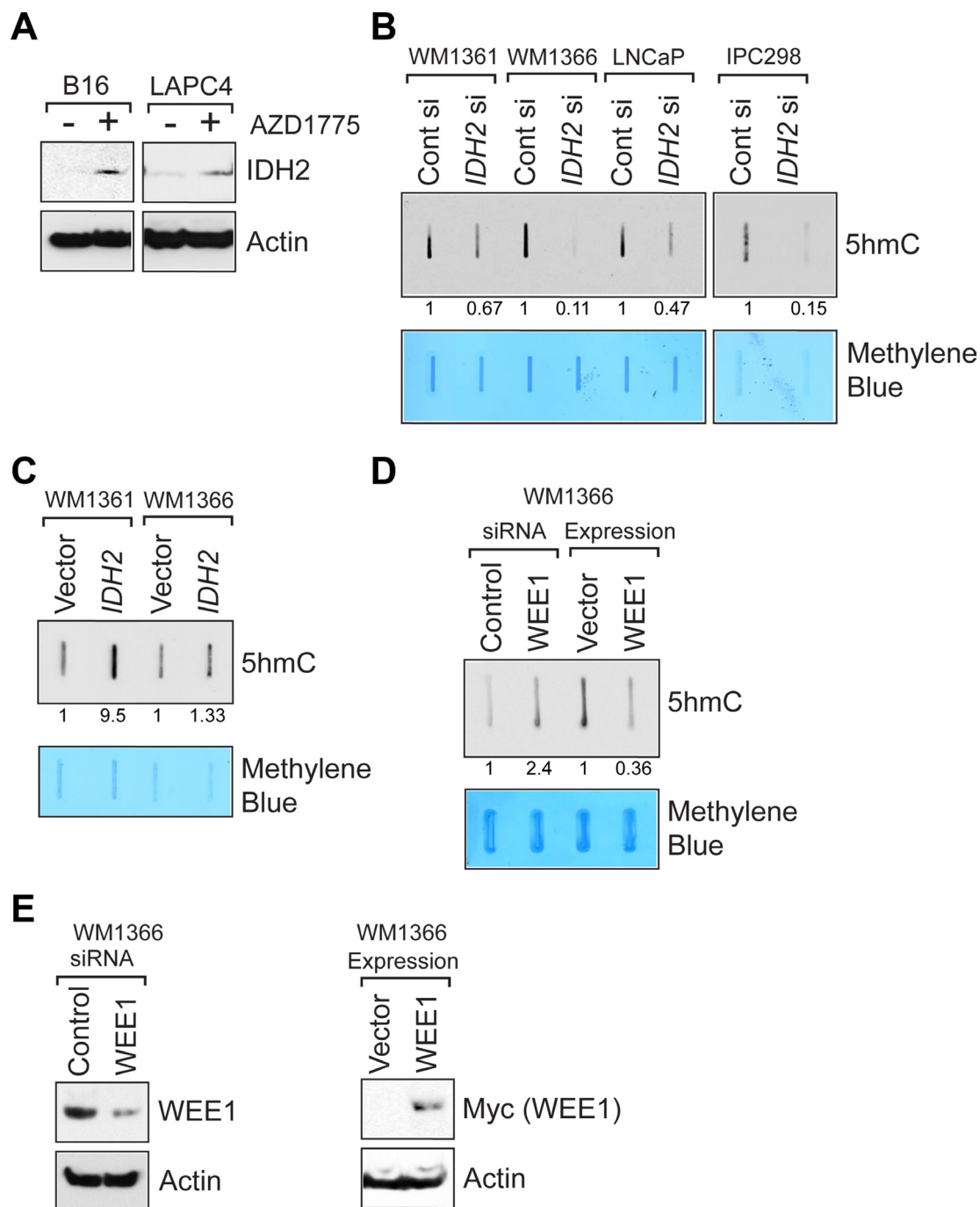


Figure 4: WEE1 negatively regulates *IDH2* protein expression. (A) Melanoma (B16) and prostate cancer (LAPC4) cells were treated with AZD1775 (2 μ M, 24 hr) and lysates were immunoblotted with anti-IDH2 antibodies. Lower panel indicates immunoblotting of lysates with actin antibodies. (B) Melanoma (WM1361 and WM1366) and prostate cancer (LNCaP) derived cells were transfected with IDH2 siRNA and total genomic DNA was isolated. Equal amount of DNA was immobilized onto nytran membrane, followed by immunoblotting with 5-hmC antibodies. Numbers shown below top panel indicates relative intensity of the bands compared to untreated controls. Lower panel indicates methylene blue staining of the same blot to confirm equal loading of the DNAs. (C) Melanoma cells were transfected with human IDH2 expressing construct and total genomic DNA was isolated. Equal amount of DNA was immobilized onto nytran membrane, followed by immunoblotting with anti-5-hmC antibodies. Numbers shown below top panel indicates relative intensity of the bands compared to untreated controls. Lower panel indicates methylene blue staining of the same blot to confirm equal loading of the DNAs. (D) WM1366 cells were transfected with either WEE1 or control siRNA or human WEE1 (Myc-tagged) expressing construct. Total genomic DNA was isolated. Equal amount of DNA was immobilized onto nytran membrane, followed by immunoblotting with anti-5-hmC antibodies. Numbers shown below top panel indicates relative intensity of the bands compared to untreated controls. Lower panel indicates methylene blue staining of the same blot to confirm equal loading of the DNAs. (E) WM1366 cells were transfected with either WEE1 or control siRNA or human WEE1 (Myc-tagged) expressing construct. Lysates were immunoblotted with indicated antibodies.

GBMs display elevated WEE1 and a significant decrease in *IDH2* mRNA expression profiles

Based on our finding that WEE1 epigenetically regulates the expression of chromatin modifying gene *IDH2*, we envisaged that alterations in WEE1 regulatory control could significantly disrupt the epigenetic landscape of GBMs, where increased WEE1 expression has been

noted. To interrogate this hypothesis further, we analyzed 27 primary grade IV glioblastomas and 6 normal brain samples. All tumors were microdissected and a pathologist validated each of the tissue samples prior to its usage. Total RNA was prepared, followed by qRT-PCR using *WEE1*, *IDH2* and Actin specific primers. A significant increase in WEE1 mRNA levels is observed in GBM tumors compared to the normal human brain samples

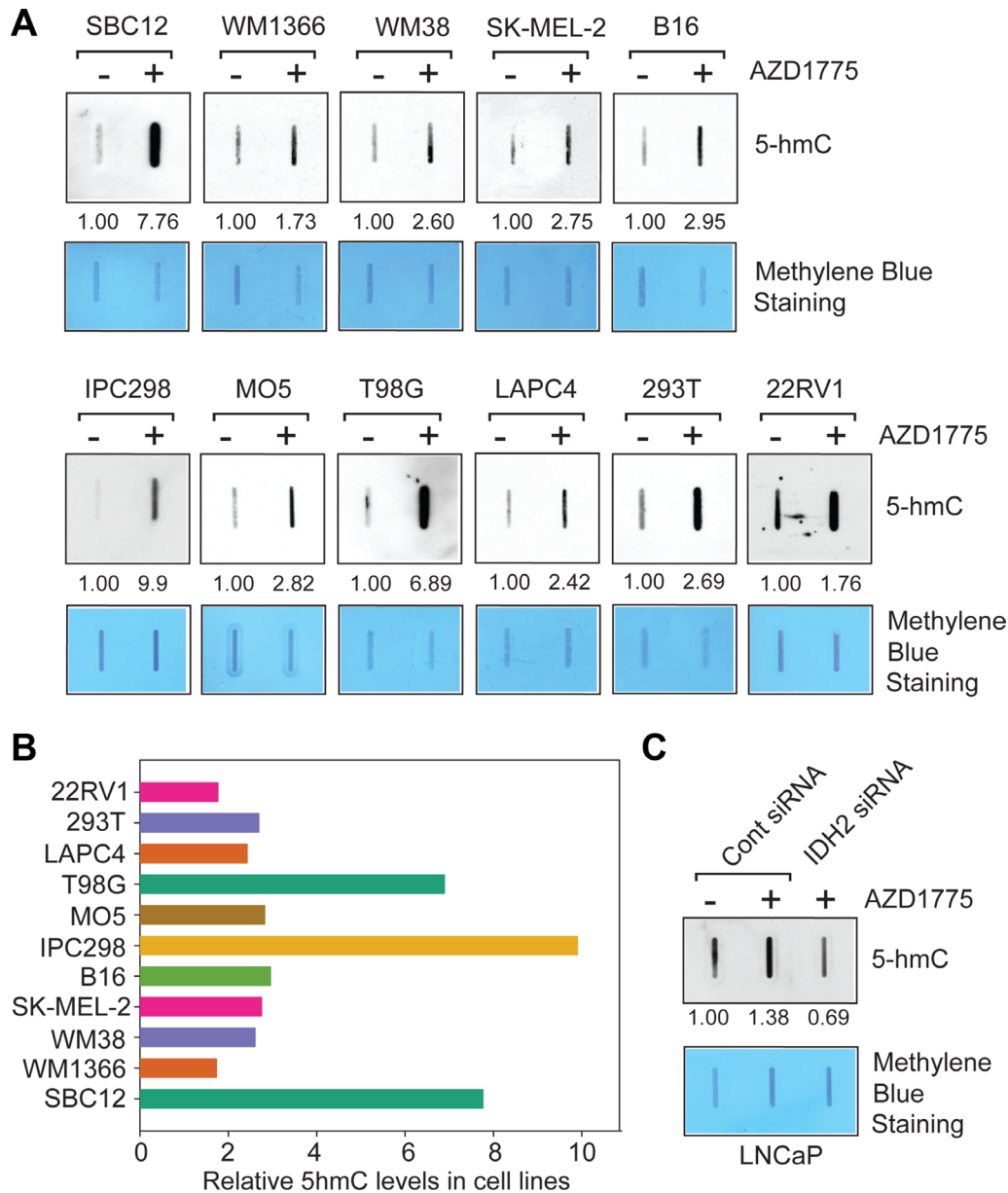


Figure 5: Inhibition of WEE1 kinase activity causes upregulation of 5-hmC levels. (A) Melanoma, GBM and prostate cancer derived cells were treated with AZD1775 (1 μ M, 24 hr) and total genomic DNA was prepared. Equal amount of DNA was immobilized onto nytran membrane, followed by immunoblotting with 5-hmC antibodies. Numbers shown below top panel indicates intensity of the bands compared to untreated controls. Loading of equal amount of DNAs was confirmed by methylene blue staining (lower panels). (B) Relative 5-hmC levels in AZD1775 treated cells are shown in comparison to untreated cells. (C) LNCaP cells were transfected with IDH2 or control siRNA. 24 hour post transfection, cells were treated with AZD1775 (1 μ M, 24 hr) and total genomic DNA was isolated. Equal amount of DNA was immobilized onto nytran membrane, followed by immunoblotting with 5-hmC antibodies. Numbers shown below top panel indicates intensity of the bands.

(Figure 6A). In contrast to WEE1, a significant decrease in *IDH2* mRNA levels was apparent in GBMs as compared to normal brain samples (Figure 6B).

To identify patients that exhibited a characteristic WEE1/*IDH2* signature, the threshold for each variable was selected to maximize the sensitivity (ratio of positives to GBMs) when the specificity (ratio of negatives to normal samples) is set to 1. GBM patients whose WEE1/Actin and *IDH2*/Actin ratios are >0.774 and <1.27 , respectively, are likely to have highly active/elevated WEE1/*IDH2* signaling. There were 7 such patients (patient #1, 4, 9, 15, 17, 18 and 21) that fit in this category, indicating that about 26% (7 out of 27) of the GBM patients exhibit elevated WEE1/*IDH2* signaling (90% CI: 0.128–0.432). Patient #14 appears to be an outlier, wherein we observed high levels of WEE1 and *IDH2* expression. In contrast, none of the normal brain samples exhibited >0.774 and <1.27 ratios for WEE1/Actin and *IDH2*/Actin, respectively.

Metastatic melanomas display a significant decrease in *IDH2* mRNA expression

To interrogate *IDH2* gene expression levels in primary human melanomas total RNA was isolated. As a control RNA was also prepared from 6 normal skin samples and a primary human keratinocyte derived cell line. All tumors were microdissected and a pathologist validated each of the tissue samples prior to its usage. To validate a functional consequence of pY37-H2B deposition at the *IDH2* genomic locus, the relative expression of *IDH2* mRNAs was determined based on its ratio with actin. The log-transformation was taken so that the data were normally distributed. A significant decrease in *IDH2* mRNA expression profile was apparent in melanomas as compared to normal skin samples or primary human keratinocyte derived cell line (Figure 7A).

The differences in relative *IDH2* mRNA levels between melanoma and primary human keratinocyte were statistically significant ($p = 0.048$). The p -values are two-sided and computed by the two-sample t -test. To identify patients that exhibit significant downregulation of *IDH2*, the threshold for each variable was selected to maximize the sensitivity (ratio of positives to melanoma) when the specificity (ratio of negatives to normal samples) is set to 1. These indicated that the melanoma patients whose *IDH2*/Actin ratios are <1.8 , are likely to have active/elevated WEE1/pY37-H2B signaling (Figure 7A). There were 10 such patients (patient #9, 11, 12, 15, 16, 17, 18, 19, 20 and 21) that fit in this category, indicating that about 71% (10 of 14) of the melanoma patients exhibit elevated WEE1/pY37-H2B signaling (90% CI: 0.128–0.432). In contrast, two of the 6 normal skin samples (sample 3 and 4) exhibited <1.8 ratios for *IDH2*/Actin. Interestingly, 4 out of 7 (51%) WT BRAF patients exhibited a significant suppression of *IDH2* levels (Figure 7A). In addition to BRAF WT melanomas, *IDH2* levels were also assessed

in BRAF V600E mutant melanomas. 3 out of 4 (75%) mutant BRAF melanoma samples exhibited a significant downregulation of *IDH2*. These data open up an intriguing possibility that WEE1 epigenetic signaling is likely to be independent of BRAF mutational status.

To further assess the outcome of downregulation of *IDH2* transcription, 5-hmC levels were examined in skin and melanoma samples. A significant decrease in global 5-hmC levels was observed in melanomas as compared to the skin samples (Figure 7B and 7C).

Suppression of WEE1 epigenetic activity impedes melanoma xenograft tumor growth

Not only is *WEE1* overexpressed in melanomas, AZD1775, a potent WEE1-specific inhibitor selectively induces apoptosis in WEE1 expressing cancer cell lines [33–35], suggesting that some of the melanomas may be utilizing the WEE1 signaling pathway for survival. To examine whether suppression of WEE1 epigenetic activity impedes melanoma xenograft tumor growth, mouse melanoma cell line MO5 was injected in B6 mice. 10 days post-injection of the cells, when palpable tumors were noticed, mice were given AZD1775 by oral gavage at 25 mg/kg of body weight, three times a week, for 3 weeks. As a control, 10% DMSO (vehicle) was given by oral gavage. Tumor volumes were measured. Decrease in tumor volumes was observed in mice given AZD1775 by oral gavage (Figure 8A). Importantly, this treatment regimen was found to be well tolerated as mice exhibited little weight loss (Figure 8B).

To examine 5-hmC status in xenograft tumors, the tumors were excised and the genomic DNAs was prepared and analyzed. An increase in 5-hmC levels was seen in xenograft tumors isolated from mice that were injected with AZD1775, as compared to DMSO (vehicle) treated mice (Figure 8C and 8D).

DISCUSSION

The coordinated control of epigenetic signaling networks ensures proper gene expression programs that are necessary for cellular homeostasis. Although, histone tyrosine phosphorylation is relatively new and understudied epigenetic modification, it has been shown to be involved in cellular processes of high importance. For example, pY37-H2B epigenetic marks were involved in regulating global histone synthesis [7], while pY88-H4 marks regulated androgen receptor expression in the absence of its ligand [14, 36]. Surprisingly, we observed that an epigenetic modification e.g. histone modification regulates another epigenetic modification, a DNA methylation, indirectly via modulating a metabolic and tumor suppressor gene, *IDH2*. These results suggested that the cancer cells could benefit enormously if they were to hijack the WEE1/pY37-H2B epigenetic signaling

to regulate DNA methylation in cells. To address this supposition, we examined the mRNA levels of *WEE1*, *IDH2* and Actin (control) in melanoma and GBM tumors and observed that these patients exhibited elevated *WEE1* mRNA expression and a significant down regulation of *IDH2* transcription (Figure 6). Moreover, ChIP-PCR revealed that pY37-H2B marks were deposited within the *IDH2* coding region indicating a chromatin control of *WEE1* transcription (Figure 2). Conversely, inhibition of *WEE1* with the specific inhibitor, AZD1775 increased *IDH2* transcript levels and restored 5-hmC levels (Figures 3–5). This is an important finding, as reversal of the 5-hmC levels by *WEE1* inhibitor (or any other

inhibitor) has not been previously demonstrated. Given the emergence of loss of *IDH2* as an important event in various malignancies, these results suggest that cancer cells overexpressing *WEE1* may epigenetically suppress the expression of *IDH2* gene to prevent the formation of 5-hmC (Figure 9). Resulting changes in its cognate epigenetic landscape, may promote gene expression programs that favor tumor growth.

BRAF mutations, particularly at codon 600, are found in almost 50% of the cutaneous melanomas [37], which has led to the successful treatment using BRAF-selective inhibitors, vemurafenib, and dabrafenib, resulting in a prolongation of progression-free and overall

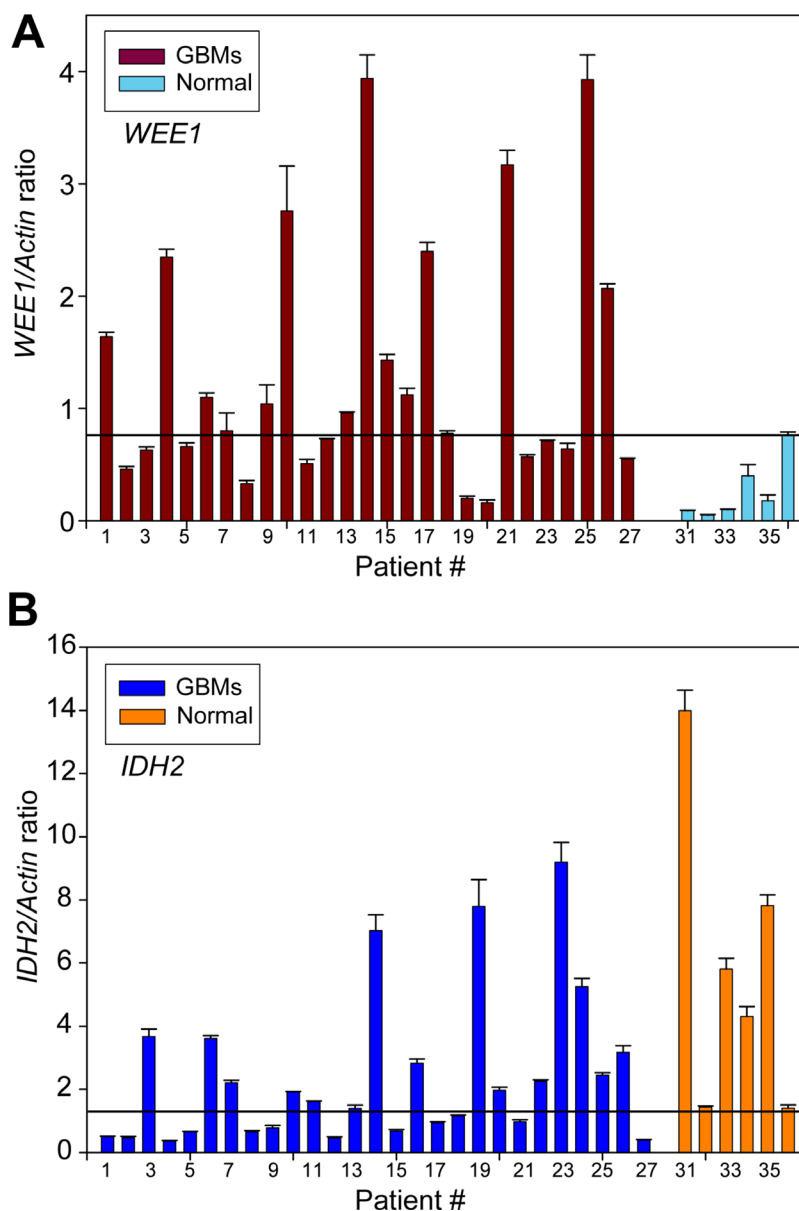


Figure 6: Upregulation of *WEE1* and downregulation of *IDH2* mRNA expression profiles in GBM patients. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *WEE1* (A) and *IDH2* (B) and actin mRNAs in GBM tumors and normal brain samples. Data represent mean $\pm$ standard error of the mean (s.e.m.) ($n = 3$).

survival. Unfortunately, limited treatment options are available for the remaining 50% of the melanoma patients whose tumors are wild-type for BRAF (WT BRAF), with a median overall survival of less than one year [38]. Recent success with PD-1 and PD-L1 antibodies has been encouraging, however further work needs to be undertaken to investigate the possibility of stratifying patients to predict whether or not they will respond to PD-1 therapy. Given the high prevalence of WT BRAF in melanomas, we reasoned that melanomas with wild-type BRAF may exhibit a dependence on WEE1 epigenetic pathway activation for maintenance of the transformed phenotype. Interestingly, the five melanoma cell lines WM1963, IPC298, WM1396, WM3918, SK-MEL-2

that do not possess V600E mutation responded well to the WEE1 inhibitor, increasing *IDH2* mRNA and 5-hmC levels. Further, MO5, a mouse melanoma cell line with WT BRAF too responded to AZD1775, showing tumor regression (Figure 8A). Moreover, 4 of 7 melanoma patients with WT BRAF too exhibited significant downregulation of *IDH2* levels. Taken together, these data indicate that WEE1 epigenetic inhibitor could have a significant impact on treatment of melanomas lacking V600 mutation, a critical unmet clinical need.

IDH1 and *IDH2* mutations have been identified in ~15–20% of AML and glioma patients^{33,34}. These mutations are missense changes. As a result of these mutations, the mutant *IDH1* and *IDH2* acquire a novel

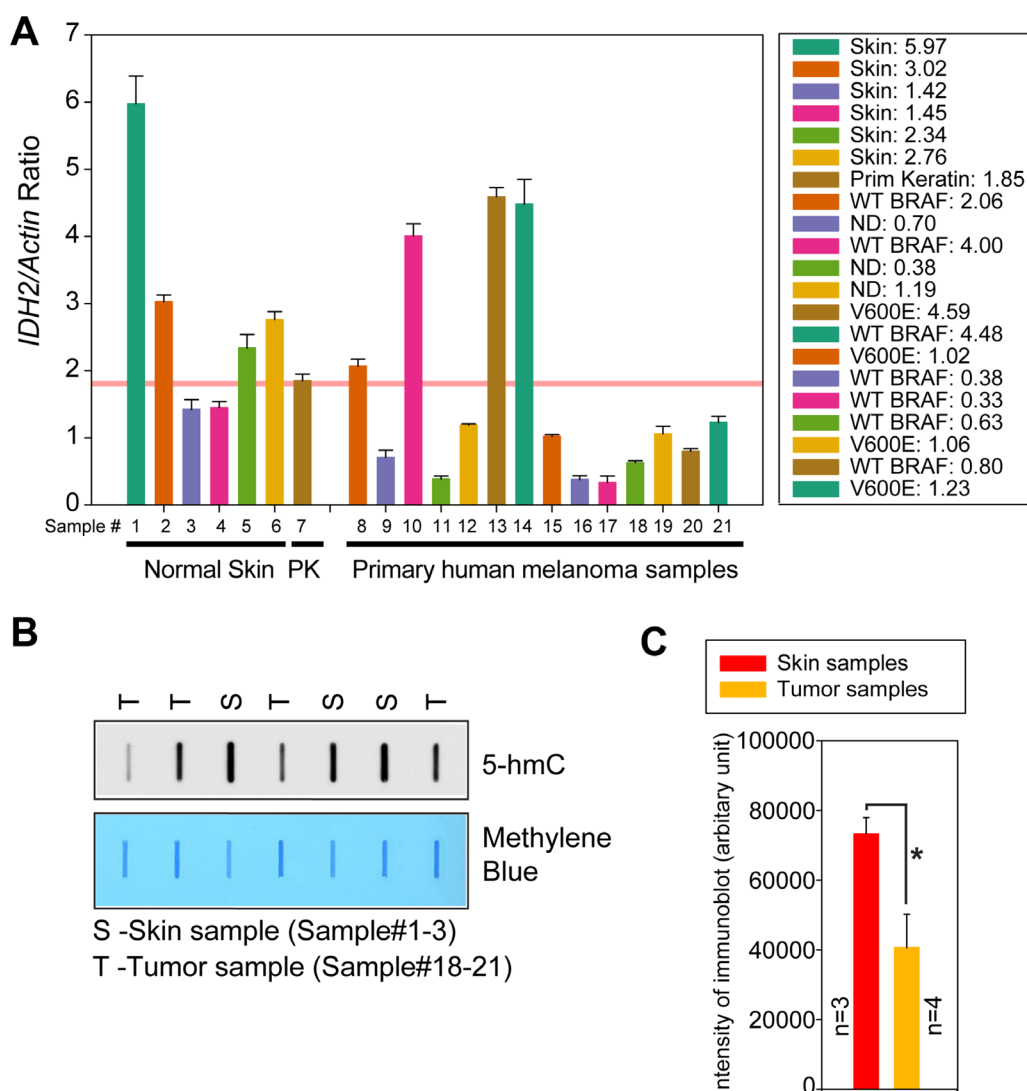


Figure 7: Downregulation of *IDH2* mRNA expression in melanoma patient samples. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *IDH2* and actin RNAs in normal skin samples, primary keratinocytes (PK) and melanoma tumors. Data represent mean $\pm$ s.e.m. ND, BRAF status not determined. (B) Total genomic DNA was isolated from melanoma and skin samples. Equal amount of DNA was immobilized onto the nytran membrane, followed by immunoblotting with 5-hmC antibodies. Loading of equal amount of DNAs was confirmed by methylene blue staining (lower panels). (C) Quantitation of the signal is shown in normal skin ($n = 3$) and tumor sample ($n = 4$).

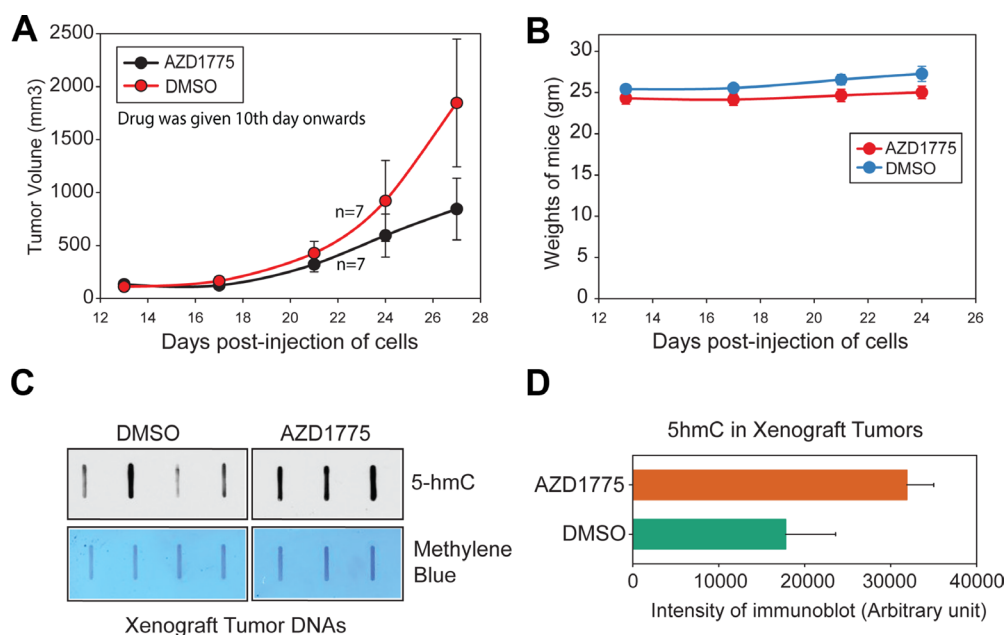


Figure 8: Inhibition of WEE1 activity compromises melanoma xenograft tumor growth. (A) MO5 melanoma cells were implanted subcutaneously in male B6 mice. When the tumors were palpable mice were given oral gavage with vehicle (10% DMSO in PBS) or AZD1775 (25 mg/kg of body) for 3 days a week ($n = 7$ mice for each treatment). Tumor volumes were measured using calipers. Mean $\pm$ s.e.m. (B) Weights of the mice are shown. (C) DNA was isolated from MO5 xenograft tumors followed by 5-hmC levels assessment by slot blot. (D) 5-hmC levels are shown in histogram.

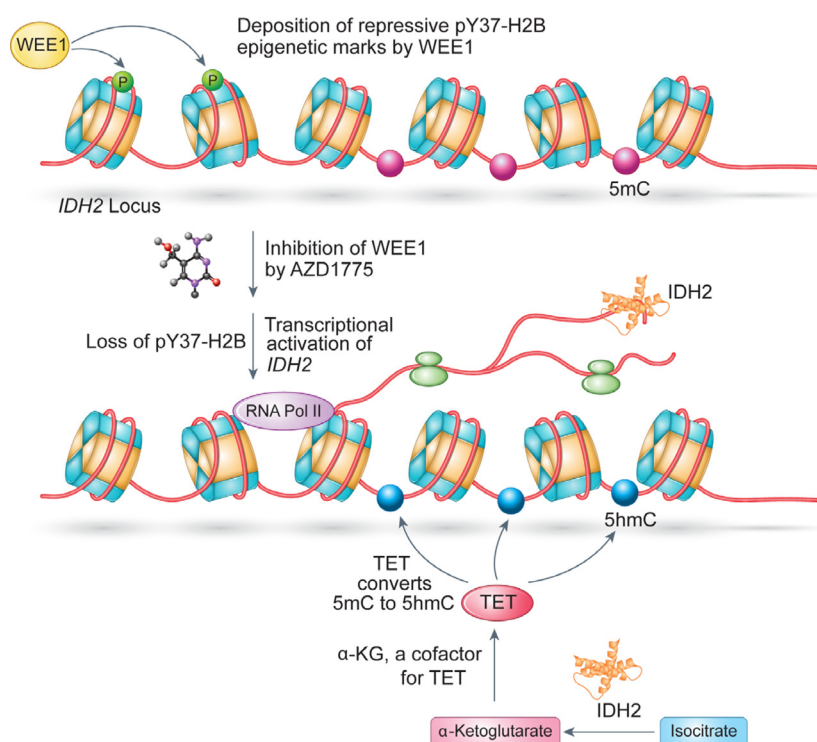


Figure 9: WEE1 pY37-H2B epigenetic signaling axis downregulates *IDH2* mRNA expression and 5-hmC levels in cancer cell; a model. WEE1 overexpressing cancer cells deposits pY37-H2B repressive marks to suppress the expression of *IDH2* transcription which is reflected in a significant loss of global 5-hmC levels that promotes gene expression programs that favor tumor growth. Inhibition of WEE1 by AZD1775 led to the loss of pY37-H2B which in turn restored 5-hmC levels, compromising cancer cell proliferation.

enzymatic activity, catalyzing the reduction of α -KG to an oncometabolite, 2-hydroxyglutarate (2-HG) [24, 39]. The 2-HG competitively inhibits α -KG-dependent enzymes such as histone demethylases and TET2, which not only resulted in an increased dimethylation of histone H3K9 and H3K79, but also caused a loss of 5hmC levels in the DNA [24, 25]. Both *IDH1* and *IDH2* mutations were identified in ~15% of gliomas, which were predominantly seen in grade II-III gliomas and secondary glioblastomas [40, 41]. Very few mutations were identified in primary GBMs (about 3–5%) [41, 42]. Interestingly, in spite of the general absence of *IDH1* and *IDH2* mutations in primary GBMs, 5-hmC levels were significantly reduced in GBMs [27, 32]. Importantly, the loss of 5-hmC in brain cancer did not exhibit any correlation with *IDH1* or *IDH2* mutations [27]. Similar 5-hmC depletion is reported in other cancers, including lung cancer, where its levels do not correlate with *IDH* mutations [32]. Furthermore, mutations in *IDH1* or *IDH2* genes have not been identified in melanomas, but still, virtually all malignant melanoma samples exhibited either a partial or complete loss of 5-hmC [26]. Collectively, these data indicate that *IDH1/2* mutations leading to an increased methylation of H3K9 and H3K79 and decreased 5-hmC may occur in a context-dependent manner in a subset of Acute Myeloid Leukemias, oligodendrogliomas and astrocytomas. Absence of *IDH1* and *IDH2* mutations in the melanoma (or low occurrence in GBMs) indicates that these mutations are unlikely to be the major determinants promoting a loss of 5-hmC in melanomas, primary GBMs and prostate cancers.

Overall, the inhibition of WEE1 erased pY37-H2B repressive marks, causing a marked increase in the levels of *IDH2*, thus reversing the 'loss of the 5-hmC' phenotype (Figure 9). Future development of WEE1 'epigenetic inhibitor' could facilitate specific targeting of many of WT BRAF melanomas, GBMs, triple negative breast cancers (TNBCs) and castration resistant prostate (CRPCs), which currently have a few therapeutic options.

MATERIALS AND METHODS

Ethics statement

Mice breeding and colony maintenance was performed according to IACUC protocols approved in writing by University of South Florida (USF) Division of Research Integrity and Compliance. We used the human tissues for our study, which were obtained from Moffitt Cancer Center under SRC/IRB approved protocol, MCC#15375, IRB Study #106509 (PI: Sarnaik). The samples were obtained in accordance with the approved guidelines. All experimental protocols were approved by Moffitt Cancer Center & USF SRC/IRB committee. A written informed consent was obtained from all subjects prior to collection of samples.

Cell lines, antibodies, inhibitors

LNCaP and VCaP cells were obtained from ATCC. GBM cell lines were provided by Peter A. Forsyth. Melanoma cell lines were obtained from laboratory of Keiran Smalley at the Moffitt Cancer Center. *IDH2* and WEE1 siRNA were obtained from Dharmacon. For most experiments, cells were treated with 1 μ M AZD1775 for 24 hr. WEE1, Actin and H2B antibodies were purchased from SantaCruz. 5hmC antibodies were purchased from Active Motif. The small molecule inhibitor, AZD1775 was purchased from Selleck Chemicals. H2B Y37-specific antibodies have been previously described [7].

Mouse xenograft studies

All animal experimentation were performed using the standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals (IACUC Protocol # 4151R). 3×10^5 MO5 cells were suspended in 200 μ l of PBS with 50% matrigel (BD Biosciences) and were implanted subcutaneously into the dorsal flank of six week old male B6 mice. Once the tumors reached approximately 100 mm³ in size (about 10 days), mice were given AZD1775 (or 10% DMSO in PBS as vehicle) at the concentrations 25 mg/kg of body weight, three times a week, for 3 weeks. Tumor volumes were measured twice weekly using calipers. At the end of the study, all mice were humanely euthanized, tumors extracted and weighed. Additionally, spleen, liver and spleen were harvested and stained with H&E to determine effect of inhibitor at the organ level.

Cell synchronization using a double thymidine block

MEFs were grown to 60–70% confluency. Thymidine is added at a final concentration of 2 mM and incubated for 17 hours. Cells were washed and fresh serum containing media was added. After 10 hours, thymidine was added again and cells were incubated for 17 hours; cells were washed, replaced with serum containing media and time points were collected.

Chromatin immunoprecipitation (ChIP) and ChIP-sequencing

Cells (5×10^7 cells) were either untreated or treated with AZD1775. Cells were harvested, fixed in p-formaldehyde and nuclei were prepared. Purified nuclei were resuspended in RLB buffer [43] and sonicated for 25 seconds. The soluble chromatin was incubated overnight at 4°C with antibodies and protein-G or A magnetic beads. The soluble chromatin was processed in the same way without immunoprecipitation and termed input DNA. The

amount of immunoprecipitated DNA was determined by real-time PCR. The complexes were washed with ChIP buffer 1 and 2 (Active Motif), eluted with elution buffer and subjected to proteinase-K treatment. ChIP DNA was subjected to proteinase-K treatment and was purified using PCR DNA purification column.

Quantitative RT-PCR and ChIP-PCR

All RT reactions were done at the same time so that the same reactions could be used for all gene studies. For the construction of standard curves, serial dilutions of pooled sample RNA were used (50, 10, 2, 0.4, 0.08, and 0.016 ng) per reverse transcriptase reaction. One “no RNA” control and one “no Reverse Transcriptase” control were included for the standard curve. Three reactions were performed for each sample: 10 ng, 0.8 ng, and a NoRT (10 ng) control. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All standards, the no template control (H₂O), the No RNA control, the no Reverse Transcriptase control, and the no amplification control (Bluescript plasmid) were tested in six wells per gene (2 wells/plate × 3 plates/gene). All samples were tested in triplicate wells each for the 10 ng and 0.8 ng concentrations. The no RT controls were tested in duplicate wells. PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) using 2 µl of cDNA (or ChIP DNA) and the primers in a 20-µl final reaction mixture. After 2-min incubation at 50°C, AmpliTaq Gold was activated by 10-min incubation at 95°C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C and hybridization of primers for 1 min at 55°C. Dissociation curves were generated for each plate to verify the integrity of the primers. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The actin data were used for normalizing the gene values; i.e., ng gene/ng actin per well. The primer sequences are shown below.

Primer sequences for qRT-PCR

Human IDH2 FP	AGATGGCAGTGGTGTCA AGGAG
Human IDH2 RP	CTGGATGGCATACTGGA AGCAG
Mouse IDH2 FP	GGCTGTCAAGTGTGCCA CAATC
Mouse IDH2 RP	TTGGCTCTCTGAAGACG GTTCC
Human ACTIN FP	CACCATTTGGCAATGAGC GGTTC
Human ACTIN RP	AGGTCTTTGCGGATGTC CACGT

Human HIST H2AI FP	CGACAACAAGAAGACT CGCATCA
Human HIST H2AI RP	TGTGCGATGGTGACTTT GCCCA

Primer sequences for ChIP-PCR

Mouse IDH2 ChIP 3	TAGACGTGAA GGCGCAACTC CGGACAAAAT
Mouse IDH2 ChIP 4	TTC CGT GGCAAC AGGCATCA AAAGAGGCCA
Human IDH2 ChIP 7	TAAGATCTGGTATGAGCACCG GCTCATTGA
Human IDH2 ChIP 8	GACCAACAGTCCACCCCCAC AGGGAGCAGC

RNA isolation from human tumors and cell lines

RNAs from normal skin samples, primary human melanomas, primary human keratinocytes prostate cancer cell lines and glioblastomas were isolated using the Trizol method followed by purification with the Qiagen RNeasy plus kit that included the genomic DNA elimination step. All the tumors were microdissected and pathologist validated each of the tissue samples prior to its usage. RNA from cell lines was isolated using RNeasy plus kit (Qiagen) that included the genomic DNA elimination step.

Genomic DNA isolation from xenograft tumors and cell lines

Total genomic DNAs from mouse xenograft tumors were isolated using ZR Genomic DNA Tissue MicroPrep (Zymoresearch, CA). Genomic DNA was isolated from cell lines using Quick gDNA MicroPrep (Zymoresearch, CA).

5-hmC analysis by slot blot

1 µg of the genomic DNA was treated with 5 µM NaOH and heated to 95°C for 5 min to denature the DNA. DNA was snap chilled, mixed with 100 µM Ammonium acetate and 0.4 µg of the DNA was blotted onto nitrocellulose membrane. DNA was UV cross-linked and immunoblotted with anti-5-hmC antibody (Active Motif).

Flow cytometry

Melanoma cell lines were treated or untreated with WEE1 inhibitor AZD1775 (3 µM) overnight for 24 h with vehicle or AZD1775 (Selleck chemicals) and harvested by gentle trypsinization. Cells were stored in 100 µl of sodium citrate buffer. Cells were trypsinized in 450 µl of trypsin solution at room temperature for 10 min, incubated in 375 µl of trypsin inhibitor/RNase A (Sigma) solution for 10 min, stained with 250 µl of ice-cold propidium iodide

solution for 10 min, and kept in the dark. Samples were analyzed using the Canto flowcytometer; 10,000 events were collected, and cell cycle analysis was carried out using the ModFit program.

Author contributions

K.M. and N.P.M. conceived and performed experiments, analyzed the data and wrote the manuscript; P.M., S.B. and V.S. performed the experiments; A.S., J.K., S.P-T and J.W. analyzed data. All the authors reviewed the manuscript.

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CONFLICTS OF INTEREST

K.M. and N.P.M. are named as inventors on a patent application no. 14/124,662 titled 'Antibodies specific for phosphorylated histones and uses thereof' and a patent application no. 14/860,405 titled 'Methods for the treatment of disorders related to phosphorylation of histones'.

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