UNIVERSITY OF CINCINNATI

Date: December 12, 2007

I, Kevin A. Link,
hereby submit this work as part of the requirements for the degree of:
Doctor of Philosophy

in:

Cell and Molecular Biology

It is entitled:

BAF57 Modulation of Androgen Receptor Action and
Prostate Cancer Progression

This work and its defense approved by:

Chair: Karen Knudsen

Sohaib Khan

Jinsong Zhang

Erik Knudsen

Bernard Weissman

Carolyn Price

BAF57 Modulation of Androgen Receptor Action and Prostate Cancer Progression

A dissertation submitted to the Division of Research and Advanced Studies at the
University of Cincinnati in partial fulfillment of the requirements for the degree of

Doctorate of Philosophy (Ph.D.)

In the Department of Cell and Cancer Biology of the College of Medicine

2007

by

Kevin A. Link

B.S. College of Mount St. Joseph, 2002

Committee Chair: Karen E. Knudsen, Ph.D.

Abstract:

Prostate cancer relies on androgens for growth and survival. Androgens elicit their function primarily through the androgen receptor (AR), a transcription factor that is required for the development and progression of prostate cancer. Therefore, current treatment options for disseminated disease are aimed at either inhibiting androgen synthesis or preventing activation of the receptor. These treatment options are initially effective; however, recurrent disease ultimately arises within two to three years that typically present as therapy resistant. From this, it is obvious that more effective means of targeting this pathway are necessary to treat prostate cancer. The present study exploited a specific aspect of the AR activation pathway and uncovered a novel means for potentially targeting AR action and subsequent prostate cancer proliferation. The SWI/SNF chromatin remodeling complex is one such necessary component involved in mediating AR activation. Specifically, the BRM core ATPase was demonstrated to exhibit a preferential response to activating AR target genes which was dependent on promoter context. Subsequent studies identified the BAF57 subunit of the SWI/SNF complex as the primary interacting AR subunit, thus indicating a possible role in complex recruitment to AR target DNA. BAF57 proved to be critical for AR mediated transcriptional activation, AR enhanced coactivator activation, and prostate cancer proliferation. Based on these findings, the potential for targeting BAF57 as a prostate cancer therapeutic option was examined. First, expression of BAF57 was verified in human prostate tissue specimens, with possible enhanced expression observed in metastatic samples.

Subsequent molecular analyses determined that the primary region of interaction occurred between the DBD/hinge region of AR and the proline-rich/HMG domain of BAF57. The N-terminal binding region of BAF57 was then shown to reduce AR recruitment to AR target genes, inhibit AR target gene activity, and decrease prostate cancer cell proliferation. Together, the data herein establish the SWI/SNF chromatin remodeling complex, specifically BAF57, as a valid target for the treatment of prostate cancer.

Acknowledgments:

The success of my graduate career could not have been possible without the constant support and superb direction I received from my mentor, Dr. Karen Knudsen. For always being willing to give advice and for the numerous words of encouragement, I am truly appreciative. The level of scientific learning was far beyond my expectations and for that I am grateful. I would also like to thank the members of my thesis committee who contributed greatly to several insightful discussions and helped shape the direction of my thesis: Dr. Erik Knudsen, Dr. Sohaib Khan, Dr. Jinsong Zhang, Dr. Carolyn Price, and Dr. Bernard Weissman. Additionally, I am thankful for all of the members of the laboratories of Karen and Erik Knudsen who provided insightful comments regarding manuscripts, presentations, etc. Thanks also for making graduate school an enjoyable experience. I would also like to thank my family and friends for their encouragement and understanding. Lastly, I thank my wife and best friend, Jen, for her never-ending love and support. She has never left my side throughout graduate school and will always be there to support me.

Table of Contents

	Page
List of Figures.....	2
Chapter I: Introduction	
A. Prostate cancer.....	4
B. Prostate cancer diagnosis and therapy.....	4
C. Androgen receptor signaling.....	5
D. AR regulation in development.....	6
E. AR regulation in prostate cancer.....	7
F. Necessity of additional molecular targets.....	8
G. Co-activator involvement in cancer.....	9
H. SWI/SNF regulation of nuclear receptor action.....	10
I. References.....	14
Chapter II: Differential Requirement of SWI/SNF for Androgen Receptor Activity	
A. Abstract.....	21
B. Introduction.....	21
C. Materials and Methods.....	22
D. Results.....	22
E. Discussion.....	26
F. References.....	28
Chapter III: BAF57 Governs Androgen Receptor Activity and Androgen- Dependent Proliferation through SWI/SNF	
A. Abstract.....	31
B. Introduction.....	32
C. Materials and Methods.....	36
D. Results.....	45
E. Discussion.....	53
F. References.....	59
Chapter IV: Targeting the BAF57 SWI/SNF Subunit in Prostate Cancer: a novel platform to control AR activity	
A. Abstract.....	80
B. Introduction.....	82
C. Materials and Methods.....	87
D. Results.....	95
E. Discussion.....	107
F. References.....	115

Chapter V: Summary and Future Directions	
A. Androgen receptor signaling as a therapeutic target.....	138
B. SWI/SNF is critical for AR activity.....	138
C. The BAF57 subunit mediates SWI/SNF dependent AR activity....	140
D. BAF57 convergence on coactivator function.....	142
E. Developing BAF57 as a therapeutic target.....	143
F. Future studies and summary.....	147
G. References.....	149

List of Figures:

Chapter I:	
Figure 1-Androgen receptor domains.....	6
Figure 2-Androgen receptor activation pathway.....	6
Figure 3-SWI/SNF activates and represses gene transcription.....	10
Figure 4-Targeting the SWI/SNF complex as a prostate cancer therapeutic.....	12
Table 1-Target systems/proteins exhibiting BAF57 involvement.....	13
Chapter II:	
Figure 1-Complete AR-dependent activation of the proximal probasin promoter requires SWI/SNF activity.....	23
Figure 2-AR dependent activation of the probasin promoter is induced by the restoration of BRG1, but preferentially by hBRM.....	24
Figure 3-Activation of the proximal PSA promoter requires hBRM- dependent SWI/SNF activity.....	25
Figure 4-The PSA enhancer region facilitates a bypass of the SWI/SNF requirement.....	26
Figure 5-The PSA enhancer does not circumvent the requirement of SWI/SNF activity on the probasin promoter.....	27
Chapter III:	
Figure 1-BAF57 is expressed in the luminal epithelia.....	70
Figure 2-BAF57 binds the androgen receptor independent of ligand.....	71
Figure 3-BAF57 is critical for androgen receptor activity.....	72
Figure 4-Abrogation of BAF57 results in reduced endogenous androgen receptor activity.....	73
Figure 5-BAF57 action is dependent upon SWI/SNF ATPase activity.....	74
Figure 6-BAF57 is recruited to the AR regulatory regions in the presence of ligand.....	75
Figure 7-BAF57 cooperates with p160 co-activators.....	76
Figure 8-BAF57 facilitates non-p160 androgen receptor	

co-activator function.....	77
Figure 9-Abrogation of BAF57 or BRM attenuates androgen receptor dependent proliferation.....	78
Chapter IV:	
Figure 1-BAF57 is highly expressed in human prostate cancer specimens.....	130
Figure 2-BAF57 is intermittently recruited to androgen responsive elements.....	131
Figure 3-SWI/SNF ATPases and BAF57 are not required for bicalutamide mediated repression of AR activity.....	132
Figure 4-BAF57 binds AR DBD/hinge region.....	133
Figure 5-Loss of C-terminal extension within AR hinge diminishes binding to BAF57 and transactivation potential.....	134
Figure 6-BAF57 N-terminus is sufficient for AR association.....	135
Figure 7-BAF57 N-terminus reduces AR residence at AREs, limits AR activity, and suppresses prostate cancer cell proliferation..	136
Supplemental Figure 1-BAF57 expression is increased in metastatic prostate cancer specimens.....	137
Chapter V:	
Figure 1-Targeting BAF57 to inhibit AR transcriptional activity.....	146

Chapter I

Introduction:

Prostate Cancer

Prostate cancer is a major health concern in the United States with an estimated 218,000 new cases diagnosed this year. Additionally, 27,000 deaths are expected to result from this disease (1). Although the reasons for prostate cancer development remain unclear, continual stimulation via the male hormone testosterone is one of the known contributors to this disease. Several risk factors exist that are known to contribute to the development of prostate cancer. One of the strongest risk factors is age (2). Few men under the age of 40 are diagnosed with this disease; however, as men get older, the chance of developing prostate cancer significantly increases with a one in six lifetime risk (3). Race is another important risk factor in prostate cancer development, wherein African American men have an enhanced risk for developing prostate cancer and are twice as likely to die from this disease as compared to other ethnicities (4). Diet is also suspected to play an important role in development, specifically, those high in fat are thought to enhance prostate cancer risk (5).

Prostate Cancer Diagnosis and Therapy

Prostate cancer diagnosis is achieved through several screening methods, including the digital rectal exam (DRE) and measurements of serum prostate specific antigen (PSA), a protein that is secreted much more abundantly in highly proliferative prostate cells (6). Upon initial diagnosis of prostate cancer, it must first be determined whether or not it is confined to the organ. Given that

prostate cancer is thought of as a slow progressing disease, one treatment option for organ confined disease is “watchful waiting,” wherein the patient is monitored frequently through DRE and PSA screening. A more active approach to treatment of localized disease includes therapies such as radical prostatectomy (removal of the prostate gland) (7) or radiation therapy (8). These treatment options, typically performed for organ-confined disease, generally result in the patient being cured of the cancer, with the risk of possible side effects (9).

The localized treatments mentioned above are obviously not fully effective in patients with non-organ confined cancer. These patients also do not respond well to traditional chemotherapeutics designed to target rapidly dividing cells, given the slow progressing nature of prostate cancer as compared to other cancers (10). Therefore, patients with metastatic disease will typically undergo androgen ablation therapy, a procedure that is based on the ground-breaking findings by Charles Huggins in 1941 demonstrating that castrating dogs with prostate cancer resulted in regression of the tumor. Essentially, this finding highlighted the dependence of prostate cancer on the male hormone testosterone (a.k.a. androgens, produced by the testis). Testosterone elicits its function through the androgen receptor (AR), a transcription factor necessary for the progression of prostate cancer.

Androgen Receptor Signaling

AR is a transcription factor classified in the steroid nuclear hormone receptor family (Figure 1), containing a distinct AF1 transactivation domain, a well conserved DNA binding domain (DBD), a hinge domain to assist the DBD in binding DNA, and a ligand binding domain (LBD) (11). Activation

Figure 1 Androgen receptor domains. Androgen receptor consists of several domains including the AF1 transactivation domain, the zinc finger DNA binding domain (DBD), the hinge domain, and the ligand binding domain (LBD).

of the AR occurs when the male hormone testosterone enters the prostatic epithelial cell where it is converted to the more potent form of androgen, dihydrotestosterone (DHT) via the enzyme 5-alpha reductase (Figure 2) (12). DHT binds with high affinity to the AR. Upon binding, the receptor is converted from its static state to its active state through release from heat shock proteins.

Figure 2 Androgen receptor activation pathway.

The receptor then forms a homodimer and translocates to the nucleus where it binds to specific DNA sequences within AR target gene promoter regions known as androgen response elements (AREs). The most

well studied AR target gene is PSA, and as mentioned above, it is the primary protein utilized for monitoring prostate cancer development and progression.

AR Regulation in Development

The active response of AR to androgens in the body regulates expression of essential genes involved in the growth and differentiation necessary for normal male development at birth and throughout puberty. Throughout this process, the role of AR in cellular differentiation is critical as demonstrated by the development of androgen insensitivity syndrome in XY individuals containing inactivated AR (11, 13). These individuals exhibit a female phenotype with internal testicles. Normal AR function in the prostate of the adult male involves regulation of gland function. The primary function of the adult prostate gland is to contribute to seminal fluid composition (10-30%) and assist in ejaculation. Contrary to its primary role in differentiation throughout development, AR function in prostate homeostasis shifts to give a more proliferative response that leads to development and progression of prostate cancer.

AR Regulation in Prostate Cancer

Due to its essential involvement in prostate cancer growth and development, treatment of non-organ confined disease is focused on directly ablating AR activity and/or targeting factors that are essential in AR mediated transcriptional activation (14). Androgen ablation therapy is generally carried out by limiting testosterone levels in the body through multiple mechanisms such as bilateral orchiectomy (surgical castration) and/or chemical castration (e.g. gonadotropin releasing hormone agonists). Additional treatments involve targeting the AR directly by using antagonists (e.g. bicalutamide) that bind to AR in place of DHT preventing its activity (15). These treatment options are initially effective; unfortunately, most patients undergo relapse in tumor formation 2 to 3

years following tumor regression, and the recurrent cancer is generally classified as therapy resistant (independent of androgens). Therefore, androgen ablation therapy is no longer effective, ultimately leading to patient mortality (16, 17). From this, it is obvious that safer, more effective treatments are necessary to enhance quality of life for patients with organ confined cancer and prolong patient survival in those with the disseminated form of this disease.

Although late stage recurrent prostate cancers typically are androgen independent, the AR generally becomes re-activated through alternative means. Inappropriate AR activation has been shown to occur via several mechanisms: 1) hyperamplification of AR (~30% of patients) allows for low levels of residual androgens to activate the receptor, 2) mutations in the AR ligand binding domain permit binding of alternative ligands such as estradiol or even AR antagonists used to treat prostate cancer patients (e.g. flutamide), 3) ligand independent mechanisms (overexpression of MAPK signaling), wherein ligand binding is no longer needed to elicit AR activation and/or 4) overexpression of AR coactivators (e.g. SRC1 and TIF2) that essentially lower the requirements of androgen for AR activation and tumor proliferation (16, 18). The dependence on AR and the ability to re-establish activity through many mechanisms would suggest that it is imperative to elucidate the process of AR mediated transcription to uncover new therapies for recurrent prostate cancer.

Necessity of Additional Molecular Targets

As indicated above, current anti-androgen therapy is generally focused on depleting the body of testosterone production or targeting the region of androgen

binding within the receptor LBD. Given the ability of prostate cancer to circumvent these therapies, current studies are centered on alternative treatment targets. Therefore, much of the current research has involved examination of the AR activation pathway and the identification of additional factors that are necessary for receptor activation. As described above, AR binds strongly to DNA through its zinc finger DNA binding domain (DBD). Once bound, a variety of transcriptional activators are recruited to initiate transcription (19, 20). Full transcriptional activation is elicited through additional factors, such as the SWI/SNF chromatin remodeling complex, which are recruited to AREs in response to DHT (21, 22). SWI/SNF acts by loosening the DNA:histone interaction thereby making the DNA accessible to basal transcription factors (23, 24). SWI/SNF function has been demonstrated to occur through several mechanisms including alterations in nucleosome structure, nucleosome sliding, dinucleosome formation, and histone octamer transfer (24, 25). Additionally, co-activators, such as members of the p160 class, are also recruited to enhance transcription and do so primarily through modification (acetylation) of lysine residues located on the histone tails (19). Similar to SWI/SNF remodeling complexes, acetylation facilitates an open chromatin state thereby rendering accessibility to the DNA. Once the DNA is accessible, the basal transcription machinery is able to carry out AR target gene transcription, ultimately leading to AR mediated PSA secretion and proliferation.

Co-activator Involvement in Cancer

As indicated above, co-activators are often aberrantly regulated in the development of cancer. Several AR co-activators have been demonstrated to be

overexpressed in prostate cancer, including the p160's SRC1 and TIF2 (16, 18). Additionally, the ARA70 AR co-activator has also been shown to be overexpressed in several prostate cancer cases as compared to benign tissue (26). Overexpression is thought to sensitize AR to low levels of androgens within the body. Additionally, SWI/SNF has been implicated as playing a significant role in contributing to cancer development. For example, loss of the SNF5 subunit (BAF47) leads to the development of rhabdoid tumor formation (27), identifying this subunit as a tumor suppressor. Re-introduction of SNF5 into deficient cell lines induced cell cycle arrest (28). Together, these data highlight a subset of the critical functions of co-activators in modulating cancer progression and demonstrate the necessity of determining specific roles for co-activators in prostate cancer progression.

SWI/SNF Regulation of Nuclear

Receptor Action

The SWI/SNF chromatin remodeling complex is essential in regulating AR target gene transcription, thus presenting itself as a potential therapeutic target (21, 29, 30). SWI/SNF is comprised of a core ATPase, either BRG1 or BRM, and several (8-11) subunits known as BRG1 associated factors (BAFs)

Figure 3 SWI/SNF activates and represses gene transcription. The SWI/SNF chromatin remodeling complex consists of a central ATPase (BRM or BRG1) and several BRG1-associated factors (BAFs). Together, the complex acts to either activate or repress target gene transcription dependent upon signaling.

(Figure 3). The ATPase utilizes the hydrolysis of ATP to act on chromatin, generating either an open chromatin state by loosening DNA:histone interaction (transcriptional activation) or a closed chromatin state by condensing the DNA interaction with histones (transcriptional repression) (24). Previous studies have identified BRM as the preferential ATPase in regulating AR target gene transcription (29, 30). Specifically, it was shown that BRM is able to enhance/initiate gene transcription of two AR target genes with significantly higher activation than in the presence of BRG1. Additionally, a promoter specific response to BRM was also observed, wherein the presence of the enhancer region located on the PSA AR target gene was able to bypass the requirement for BRM-mediated AR activation. This bypass was not observed when the enhancer region was placed on the mouse probasin AR target gene. Together, these data outline a necessary role of the SWI/SNF chromatin remodeling complex in regulating AR target gene transcription through promoter specificity and establish the possibility for targeting this complex as a means of controlling AR activity.

In contrast to the defined chromatin remodeling action of SWI/SNF, its role in prostate cancer remains unclear. Likewise, the mechanism of SWI/SNF specificity is also poorly understood. However, it is thought that the specific BAF composition that is associated with the ATPase contributes to this specificity. *In vitro* analyses revealed that only three BAFs are necessary for the specific action of chromatin remodeling, wherein BAF170, BAF155, and BAF47 were sufficient to restore ATPase-mediated activity (31). In addition, several BAFs have been

found to be involved in the regulation of nuclear receptor transcription factor function, lending the possibility of a conserved mechanism of signaling. For example, BAF250, BAF60a, and BAF57 bind directly to the glucocorticoid receptor (GR) and are speculated to act as bridging factors between the nuclear receptor and the core ATPase (32, 33). Furthermore, the BAF57 subunit of SWI/SNF directly interacts with several transcriptional activators and repressors (summarized in Table 1). Of particular interest, BAF57 was shown to interact with the estrogen receptor (ER) and is necessary for the p160 co-activators to activate ER target genes (34). Combined, these observations prompted the hypothesis that the SWI/SNF complex significantly impacts AR mediated gene transcription and subsequent prostate cancer proliferation (Figure 4).

Figure 4 Targeting the SWI/SNF complex as a prostate cancer therapeutic.

Table 1. Target systems/proteins exhibiting BAF57 involvement.

Target System/Protein	BAF57 Function/ Transcriptional Impact
ER α	Mediates p160 coactivator function on ER target genes; binds directly to full-length ER (34)
ER β	Binds directly; no role in ER β mediated transactivation (34, 35)
GR	Binds directly
BT549 breast ductal carcinoma cell line	Induces cell cycle arrest and apoptosis through up-regulation of proapoptotic factors (36)
Dnmt3a	Associated with complex (37)
Ets-2	Complexes with Ets-2, BRG1, Ini-1; acts to repress BRCA1
Rest:Co-Rest	Found in complex; acts to repress neuronal genes (38)
CD4 T-cell regulation	Involved in silencing (39)
Erythroid Kruppel-like factor (EKLF)	Associated with EKLF coactivator-remodeling complex 1 (E-RC1) to control transcriptional activation for stage specific expression of the β -globin gene (40)
Ada2 β	Interacted with and was involved in transcriptional activation of the B-cell specific transcription factor, Pax5/BSAP (41)
β -globin	In complex with PYR (SWI/SNF-related) chromatin remodeling complex, possibly involved in fetal to adult gene switching (42)

References

1. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M. J. Cancer statistics, 2007. *CA Cancer J Clin*, 57: 43-66, 2007.
2. Bostwick, D. G., Burke, H. B., Djakiew, D., Euling, S., Ho, S. M., Landolph, J., Morrison, H., Sonawane, B., Shifflett, T., Waters, D. J., and Timms, B. Human prostate cancer risk factors. *Cancer*, 101: 2371-2490, 2004.
3. Hayat, M. J., Howlader, N., Reichman, M. E., and Edwards, B. K. Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) Program. *Oncologist*, 12: 20-37, 2007.
4. Reddy, S., Shapiro, M., Morton, R., Jr., and Brawley, O. W. Prostate cancer in black and white Americans. *Cancer Metastasis Rev*, 22: 83-86, 2003.
5. Hayes, R. B., Ziegler, R. G., Gridley, G., Swanson, C., Greenberg, R. S., Swanson, G. M., Schoenberg, J. B., Silverman, D. T., Brown, L. M., Pottern, L. M., Liff, J., Schwartz, A. G., Fraumeni, J. F., Jr., and Hoover, R. N. Dietary factors and risks for prostate cancer among blacks and whites in the United States. *Cancer Epidemiol Biomarkers Prev*, 8: 25-34, 1999.
6. Loeb, S. and Catalona, W. J. Prostate-specific antigen in clinical practice. *Cancer Lett*, 249: 30-39, 2007.
7. Damber, J. E. and Khatami, A. Surgical treatment of localized prostate cancer. *Acta Oncol*, 44: 599-604, 2005.

8. Kirkpatrick, J. P. and Anscher, M. S. Radiotherapy for locally recurrent prostate cancer. *Clin Adv Hematol Oncol*, 3: 933-942, 2005.
9. McLeod, D. G. Success and failure of single-modality treatment for early prostate cancer. *Rev Urol*, 6 *Suppl* 2: S13-19, 2004.
10. Culine, S. and Droz, J. P. Chemotherapy in advanced androgen-independent prostate cancer 1990-1999: a decade of progress? *Ann Oncol*, 11: 1523-1530, 2000.
11. Gelmann, E. P. Molecular biology of the androgen receptor. *J Clin Oncol*, 20: 3001-3015, 2002.
12. Brinkmann, A. O., Blok, L. J., de Ruiter, P. E., Doesburg, P., Steketee, K., Berrevoets, C. A., and Trapman, J. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol*, 69: 307-313, 1999.
13. Quigley, C. A., De Bellis, A., Marschke, K. B., el-Awady, M. K., Wilson, E. M., and French, F. S. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev*, 16: 271-321, 1995.
14. Balk, S. P. Androgen receptor as a target in androgen-independent prostate cancer. *Urology*, 60: 132-138; discussion 138-139, 2002.
15. Denmeade, S. R. and Isaacs, J. T. A history of prostate cancer treatment. *Nat Rev Cancer*, 2: 389-396, 2002.
16. Feldman, B. J. and Feldman, D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*, 1: 34-45, 2001.
17. Jenster, G. The role of the androgen receptor in the development and progression of prostate cancer. *Semin Oncol*, 26: 407-421, 1999.

18. Taplin, M. E. and Balk, S. P. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem*, 91: 483-490, 2004.
19. Heinlein, C. A. and Chang, C. Androgen receptor (AR) coregulators: an overview. *Endocr Rev*, 23: 175-200, 2002.
20. Shang, Y., Myers, M., and Brown, M. Formation of the androgen receptor transcription complex. *Mol Cell*, 9: 601-610, 2002.
21. Huang, Z. Q., Li, J., Sachs, L. M., Cole, P. A., and Wong, J. A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. *Embo J*, 22: 2146-2155, 2003.
22. Klok, T. I., Kurys, P., Elbi, C., Nagaich, A. K., Hendarwanto, A., Slagsvold, T., Chang, C. Y., Hager, G. L., and Saatcioglu, F. Ligand-specific dynamics of the androgen receptor at its response element in living cells. *Mol Cell Biol*, 27: 1823-1843, 2007.
23. Urnov, F. D. and Wolffe, A. P. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). *Oncogene*, 20: 2991-3006, 2001.
24. Narlikar, G. J., Fan, H. Y., and Kingston, R. E. Cooperation between complexes that regulate chromatin structure and transcription. *Cell*, 108: 475-487, 2002.
25. Flaus, A. and Owen-Hughes, T. Mechanisms for ATP-dependent chromatin remodelling. *Curr Opin Genet Dev*, 11: 148-154, 2001.

26. Hu, Y. C., Yeh, S., Yeh, S. D., Sampson, E. R., Huang, J., Li, P., Hsu, C. L., Ting, H. J., Lin, H. K., Wang, L., Kim, E., Ni, J., and Chang, C. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. *J Biol Chem*, 2004.
27. Versteeg, I., Sevenet, N., Lange, J., Rousseau-Merck, M. F., Ambros, P., Handgretinger, R., Aurias, A., and Delattre, O. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature*, 394: 203-206, 1998.
28. Betz, B. L., Strobeck, M. W., Reisman, D. N., Knudsen, E. S., and Weissman, B. E. Re-expression of hSNF5/INI1/BAF47 in pediatric tumor cells leads to G1 arrest associated with induction of p16ink4a and activation of RB. *Oncogene*, 21: 5193-5203, 2002.
29. Marshall, T. W., Link, K. A., Petre-Draviam, C. E., and Knudsen, K. E. Differential requirement of SWI/SNF for androgen receptor activity. *J Biol Chem*, 278: 30605-30613, 2003.
30. Inoue, H., Furukawa, T., Giannakopoulos, S., Zhou, S., King, D. S., and Tanese, N. Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. *J Biol Chem*, 277: 41674-41685, 2002.
31. Phelan, M. L., Sif, S., Narlikar, G. J., and Kingston, R. E. Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. *Mol Cell*, 3: 247-253, 1999.

32. Nie, Z., Xue, Y., Yang, D., Zhou, S., Deroo, B. J., Archer, T. K., and Wang, W. A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. *Mol Cell Biol*, 20: 8879-8888, 2000.
33. Hsiao, P. W., Fryer, C. J., Trotter, K. W., Wang, W., and Archer, T. K. BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. *Mol Cell Biol*, 23: 6210-6220, 2003.
34. Belandia, B., Orford, R. L., Hurst, H. C., and Parker, M. G. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *Embo J*, 21: 4094-4103, 2002.
35. Garcia-Pedrero, J. M., Kiskinis, E., Parker, M. G., and Belandia, B. The SWI/SNF chromatin remodeling subunit BAF57 is a critical regulator of estrogen receptor function in breast cancer cells. *J Biol Chem*, 281: 22656-22664, 2006.
36. Wang, L., Baiocchi, R. A., Pal, S., Mosialos, G., Caligiuri, M., and Sif, S. The BRG1- and hBRM-associated factor BAF57 induces apoptosis by stimulating expression of the cylindromatosis tumor suppressor gene. *Mol Cell Biol*, 25: 7953-7965, 2005.
37. Datta, J., Majumder, S., Bai, S., Ghoshal, K., Kutay, H., Smith, D. S., Crabb, J. W., and Jacob, S. T. Physical and functional interaction of DNA methyltransferase 3A with Mbd3 and Brg1 in mouse lymphosarcoma cells. *Cancer Res*, 65: 10891-10900, 2005.

38. Battaglioli, E., Andres, M. E., Rose, D. W., Chenoweth, J. G., Rosenfeld, M. G., Anderson, M. E., and Mandel, G. REST repression of neuronal genes requires components of the hSWI.SNF complex. *J Biol Chem*, 277: 41038-41045, 2002.
39. Chi, T. H., Wan, M., Zhao, K., Taniuchi, I., Chen, L., Littman, D. R., and Crabtree, G. R. Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature*, 418: 195-199, 2002.
40. Armstrong, J. A., Bieker, J. J., and Emerson, B. M. A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF in vitro. *Cell*, 95: 93-104, 1998.
41. Barlev, N. A., Emelyanov, A. V., Castagnino, P., Zegerman, P., Bannister, A. J., Sepulveda, M. A., Robert, F., Tora, L., Kouzarides, T., Birshtein, B. K., and Berger, S. L. A novel human Ada2 homologue functions with Gcn5 or Brg1 to coactivate transcription. *Mol Cell Biol*, 23: 6944-6957, 2003.
42. O'Neill, D., Yang, J., Erdjument-Bromage, H., Bornschlegel, K., Tempst, P., and Bank, A. Tissue-specific and developmental stage-specific DNA binding by a mammalian SWI/SNF complex associated with human fetal-to-adult globin gene switching. *Proc Natl Acad Sci U S A*, 96: 349-354, 1999.

Chapter II:

Differential Requirement of SWI/SNF for Androgen Receptor Activity

Differential Requirement of SWI/SNF for Androgen Receptor Activity*

Received for publication, May 1, 2003, and in revised form, May 28, 2003
Published, JBC Papers in Press, May 29, 2003, DOI 10.1074/jbc.M304582200

Thomas W. Marshall, Kevin A. Link, Christin E. Petre-Draviam‡, and Karen E. Knudsen§

From the Department of Cell Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0521

The androgen receptor (AR) is a ligand-dependent transcription factor whose activity is required for prostate cancer proliferation. Because ablation of AR activity is a critical goal of prostate cancer therapy, much emphasis has been placed on understanding the accessory proteins that regulate AR function in the prostate. Several co-activators have been shown to be required for full AR activity, including histone acetyltransferases and TRAP/mediator complexes. SWI/SNF comprises a family of large, multisubunit complexes present in the cell, which contain one of two core ATPases required for nucleosome re-positioning, BRG1 or hBRM. We investigated the specific requirement of the SWI/SNF core ATPases for AR function. Using cells deficient in both BRG1 and hBRM, we show that activation of one AR target promoter, prostate-specific antigen (PSA), requires SWI/SNF chromatin remodeling for activity. A second AR target promoter, probasin, maintained a low level of activation in the absence of SWI/SNF. AR stimulation on the probasin core promoter could be partially induced with BRG1, but hBRM strongly stimulated AR activity. The PSA promoter was only induced by the restoration of hBRM. In contrast, ligand-dependent activation of the estrogen receptor was equally stimulated by BRG1 or hBRM. We demonstrate that the addition of a known enhancer region to the core PSA promoter bypasses the requirement for SWI/SNF on the PSA promoter, indicating that elements upstream of specific proximal promoters can impact the influence of the SWI/SNF complex on target gene activation. Addition of the enhancer to the probasin core promoter failed to impact the SWI/SNF requirement. In summary, SWI/SNF function potentially regulates core AR target gene promoter activation, with a preference for hBRM-containing complexes. These studies highlight a role for the enhancer in altering the impact of SWI/SNF action and suggest a disparity in AR target genes for SWI/SNF requirement.

The androgen receptor (AR)¹ is a ligand-dependent transcription factor that is a member of the nuclear receptor superfamily (1–5). The AR functions to direct male sexual differen-

tiation, prostate development, and growth of both normal and neoplastic prostates (3, 6). The dependence of prostatic adenocarcinomas on AR activity forms the basis for main line treatment of non-organ confined prostate cancer. Current therapies for the disseminated disease target AR activity, as achieved through either blockade of ligand production (androgen ablation therapy) or through direct disruption of AR activity (*e.g.* bicalutamide) (7–13). Although these methods are initially effective, recurrent tumors ultimately arise that lead to patient morbidity. The vast majority of such recurrent tumors contains inappropriately activated AR (1, 2, 14–16). Restoration of AR activity following androgen ablation is known to occur through multiple mechanisms, including AR amplification, mutation of the AR ligand binding domain (thus allowing activation by non-androgenic steroids), and AR activation through alternate signal transduction pathways (3, 17). In addition, it has also been shown that AR accessory proteins are de-regulated in prostate cancer. For example, the co-activators SRC-1 and TIF2, members of the p160 histone acetyltransferase (HAT) family, are amplified in prostate cancer and are thought to contribute to inappropriate AR activation (18). In light of these observations, there is an intensive focus on elucidating the role of co-activators in regulating AR activity.

In general, AR co-activators serve to stabilize the receptor complex, promote DNA unwinding, and/or recruit the RNA polymerase II complex (19, 20). Type I co-activators, such as SRC-1, TIF2, and pCAF/p300, are recruited by agonist-bound AR. These co-activators either harbor intrinsic HAT activity or recruit HATs to the AR complex. It is believed that the acetylation of lysine residues on core histones loosens chromatin and permits access for transcription factors to bind and activate promoters (19, 21–24). Type II co-activators stabilize the ligand bound conformation of the AR homodimer, which leads to increased activation of androgen responsive genes. For example, the type II co-activator ARA70 stabilizes N- to C-terminal interactions of the androgen receptor, dependent on ligand binding. The increased stability caused by co-activator binding promotes AR activity (19, 25, 26). Lastly, the TRAP/mediator complexes enhance ligand-dependent AR activity, likely through direct recruitment of RNA polymerase II (27). Several other cofactors have been identified, but the mechanisms by which they modulate AR activity have yet to be determined.

For several transcription factors, including selected nuclear receptors, the SWI/SNF ATP-dependent chromatin-remodeling complexes have been implicated in target gene regulation (28–37). The SWI/SNF family encompasses a number of large, multisubunit complexes comprising eight or more proteins. Invariably, the SWI/SNF complex contains one of two core

* This work was supported in part by Department of Defense Grant DAMD-02-1-0037 (to K. E. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Albert J. Ryan Foundation and the University of Cincinnati Distinguished Graduate Award.

§ To whom correspondence should be addressed. Tel.: 513-558-7371; Fax: 513-558-4454; E-mail: Karen.Knudsen@UC.edu.

¹ The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; DHT, dihydrotestosterone; PSA, prostate-specific antigen; FBS, fetal bovine serum; CDT, charcoal-dex-

tran treated; RB, retinoblastoma tumor suppressor protein; HAT, histone acetyltransferase; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CMV, cytomegalovirus; E2, 17 β -estradiol; dn, dominant negative; ARE, androgen response element.

ATPases, BRG1 or hBRM. These complexes regulate the activity of transcription factors by re-organizing chromatin structure through either facilitating nucleosome condensation (which induces transcriptional repression) or nucleosome dispersion (assisting in transcriptional activation) (38–43). For example, we and others have shown that activity of the retinoblastoma tumor suppressor protein, RB, requires SWI/SNF activity for transcriptional repression of key cell cycle targets, including cyclin A (44–50). Loss of SWI/SNF activity abrogates RB-mediated transcriptional repression and the ability of RB to arrest cellular proliferation. In contrast, RB enhances association of the glucocorticoid receptor (GR) with either BRG1 or hBRM, thus resulting in increased GR activity (51–53). The association between BRG1 or hBRM and GR is known to be direct, and recruitment of SWI/SNF complexes to GR may actually stimulate displacement of GR from chromatin, allowing GR to return to the remodeled chromatin with additional transcription factors (30). Even though SWI/SNF activity clearly is necessary to repress RB targets and activate GR targets, both events can induce a cessation of cellular proliferation (cell cycle arrest and/or apoptosis) (44–46, 50, 52–54). Collectively, these observations contribute to the current hypothesis that the SWI/SNF complex can interact with sequence specific transcription factors to either promote or repress target gene activation, dependent on promoter context and complex content.

In addition to the GR, SWI/SNF activity has been shown to be essential for estrogen receptor, ER α , activity. ER α fails to activate estrogen-responsive elements in SWI/SNF-defective cells, as demonstrated by transient reporter assay. Using this same system, ER α activity was restored upon co-expression of BRG1 (33, 34). The hypothesis that ER α requires BRG1 activity was further substantiated by the observations that BRG1 can be detected at endogenous ER target promoters in the presence of ligand and that a BRG1-associated factor, BAF57, enhances ER α /SRC-1 activation of estrogen-responsive promoters (35). Presumably, such induction of ER activity contributes to ER signaling and ER-dependent proliferation in mammary epithelia and carcinoma cells.

Here, we probed the importance of SWI/SNF activity for AR function. We show that complete ligand-dependent activation of two distinct AR target promoters (PSA and probasin) requires SWI/SNF function. For PSA, SWI/SNF activity was essential, whereas for probasin low level activation was observed in the absence of SWI/SNF, indicating that this target can be at least partially activated independently of chromatin remodeling. Both core promoters were strongly stimulated by the restoration of hBRM. The re-introduction of BRG1 weakly activated the probasin promoter but was unable to stimulate the transcription of the PSA promoter by the AR. We also demonstrate that an ER-responsive promoter can be equally activated by either BRG1 or hBRM. These data suggest a strong preference for hBRM as the core ATPase for AR activity. Lastly, we report that the addition of a distal enhancer region to the proximal promoters relieved SWI/SNF dependence for PSA but did not impact the probasin promoter in remodeling-dependent target promoters. Thus, upstream elements can influence the requirement of SWI/SNF factors for AR action. Collectively, the data contained herein establish a role for hBRM-containing SWI/SNF complexes on AR activity. Moreover, these data demonstrate a role of enhancer regions in controlling the SWI/SNF requirement and reveal a disparity in AR target promoters with regard to the chromatin-remodeling requirement.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—The pSG5AR wild type androgen receptor expression plasmid was kindly provided by Dr. Chawnsang Chang

(University of Rochester, Rochester, NY) (25). The CMV- β -galactosidase construct was the gift of Dr. Jean Wang (University of California at San Diego, La Jolla, CA). The PSA61-luc reporter was kindly provided by Dr. Kitty Cleutjens (Erasmus Universiteit, Netherlands) and has been previously described (55). pcDNA 3.1 was purchased from Invitrogen. The pBJ5-BRG1 plasmid was provided by Dr. S. Goff (Columbia University, New York, NY) (56). The pCG-hBRM plasmid was given to us by Dr. M. Yaniv (Institut Pasteur, Paris, France) (29). The pBabe-dnBRG1 was a gift from Dr. B. Weissman (University of North Carolina, Chapel Hill, NC). pBS3X-ERE-luc and pCMV5-hER α were kindly provided by Dr. S. Khan (University of Cincinnati, Cincinnati, OH). Probasin-luc (ARR2-luc) was constructed from ARR2-PB, a gift of Dr. R. Matusik (Vanderbilt University) (57). Briefly, ARR2-PB (57) was digested with *Kpn*I and *Bam*HI. The probasin fragment was subcloned into the *Kpn*I and *Bgl*II sites in pGL2-Basic. PSA-luc was provided by Dr. A. Puga (University of Cincinnati) and was generated by digesting PSA61-luc (55) with *Bgl*II and *Hind*III. The PSA promoter was inserted into the *Bgl*II and *Hind*III sites in pGL3-Basic (Promega). ARR2+E-luc was constructed by inserting of the 1.3-kb *Eco*RV fragment of the PSA enhancer into the *Sma*I site of ARR2-luc. Dihydrotestosterone (DHT) was purchased from Sigma (St. Louis, MO). β -Tubulin, BRG1, and hBRM antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CD44 antibody was a gift from Dr. L. Sherman (Oregon Health Sciences, Portland, OR).

Cell Culture and Treatment—SW13, C33A, and CV1 cell lines were obtained from ATCC and maintained in a 37 °C, 5% CO₂ incubator. SW13, C33A, and CV1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Mediatech, Herndon, VA). For SW13, C33A, and CV1 reporter assays, 10% charcoal dextran-treated FBS (CDT, HyClone Laboratories, Logan, UT) and phenol red-free Dulbecco's modified Eagle's medium were utilized.

Transfection and Reporter Assays—SW13, C33A, and CV1 cells were transfected in charcoal dextran-treated (CDT) serum, which lacks steroid hormones, with the indicated plasmid constructs using the BES/calcium phosphate protocol (58). 0.75 μ g of receptor expression plasmid (SG5-AR or ER α), 0.5 μ g of reporter construct (ARR2-luc, PSA-luc, PSA61-luc, ARR2+E-luc, or 3X-ERE-luc), 2.65 μ g of either pBJ5-BRG1 or pCG-hBRM, 0.1 μ g of CMV- β -galactosidase, and empty vector (pcDNA3.1) to a total of 4 μ g were transfected for each well of a 6-well dish. Following the transfection, the cells were washed with sterile phosphate-buffered saline. SW13, C33A, or CV1 cells were then allowed to recover for a period of 5–6 h and then supplemented with 0.1 nM DHT (Sigma) or 0.1% ethanol vehicle (EtOH) for 20–24 h. Following stimulation, cells were harvested and luciferase activity was quantified using the Promega luciferase assay kit (Promega, Madison, WI). β -Galactosidase activity was used as an internal control for transfection efficiency and was measured utilizing the manufacturer's recommended protocol for the Galacto-Star kit (Tropix, Bedford, MA). Basal activity (in the presence of ethanol vehicle) was set to "1," and relative luciferase activity is shown. Experiments were performed at least in triplicate. Averages and standard deviations are shown.

Immunoblotting— 1×10^5 C33A cells were seeded into a 6-cm dish. The cells were then transfected with 8 μ g of either pcDNA 3.1, pBJ5-BRG1, or pCG-hBRM plasmid for 12 h via BES/calcium phosphate. The cells were then washed with sterile phosphate-buffered saline. After 16 h, the cells were trypsinized and pelleted. The cell pellet was lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with a protease inhibitor mixture containing 10 μ g/ml 1,10-phenanthroline, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysis of cells proceeded on ice for 10 min. After brief sonication, lysate was clarified by centrifugation at $14,000 \times g$, 4 °C for 10 min. The supernatant was quantified by using a D_C protein assay (Bio-Rad), and 30 μ g of total protein was separated by SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore). The membrane was immunoblotted with the indicated antibodies via standard techniques, and antibodies were detected using Western Lightning chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

RESULTS

The Proximal Probasin Promoter Retains Minimal Activity in the Absence of BRG1 or hBRM—It has been previously demonstrated that the SWI/SNF complex is necessary for the function of several nuclear receptors. For example, estrogen receptor

FIG. 1. Complete AR-dependent activation of the proximal probasin promoter requires SWI/SNF activity. A, CV1 (left panel) and SW13 (right panel) cells were seeded in steroid-free media and transfected with estrogen receptor ($ER\alpha$) and 3X-ERE-luc plasmids, as described under "Experimental Procedures." The cells were then washed, allowed to recover for 4–6 h, and stimulated with either 10 nM 17 β -estradiol (E2) or 0.1% ethanol vehicle, as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase (β -gal) activities. Basal activity in the presence of ethanol vehicle was set to "1," and relative luciferase activity is shown. B, CV1 (left), SW13 (middle), and C33A (right) cells were transiently transfected with expression plasmid encoding androgen receptor (AR) and the ARR2-luc reporter at the quantities shown (in micrograms), similar to A. Cells were stimulated with 0.1 nM DHT or 0.1% ethanol for 20–24 h, and luciferase and β -galactosidase activities were measured. Basal receptor activity was set to "1," and relative luciferase activity is shown.

and glucocorticoid receptor activity is absent or significantly decreased, respectively, in cell lines that are deficient in BRG1 and hBRM, the core ATPase proteins of the SWI/SNF complex. Receptor activity can be rescued through restoration of BRG1 (29, 32–34). SW13 and C33A cells are defective in both BRG1 and hBRM, and are known to lack SWI/SNF activity (29, 59). To compare, spontaneously immortalized CV1 cells, which express both BRG1 and hBRM (data not shown) were also utilized. As shown in Fig. 1A, CV1 and SW13 cells were transfected with plasmid encoding the estrogen receptor ($ER\alpha$) and CMV- β -galactosidase (as an internal control for transfection efficiency), and the 3X-ERE-luc reporter construct. $ER\alpha$ activated the ERE reporter 4.9-fold in the presence of 10 nM 17 β -estradiol (E2) (left panel). In contrast, addition of 10 nM E2 failed to activate the 3X-ERE-luc reporter in SW13 cells (right panel). These observations are consistent with previous reports demonstrating the requirement of SWI/SNF for $ER\alpha$ activity (34).

To determine if the AR requires the SWI/SNF complex for transactivation, similar experiments were performed. Briefly, SWI/SNF-competent (CV1) or -defective (SW13 and C33A) cells were cultured for 24 h in the absence of steroid hormone (phenol red-free media supplemented with 10% CDT). Cells were then transfected with plasmid encoding the androgen receptor

(pSG5-AR), CMV- β -galactosidase, and the ARR2-luc reporter plasmid at the amounts indicated. ARR2-luc is a well characterized, AR-specific reporter construct that contains two copies of the probasin promoter in tandem and directs prostate-specific expression in transgenic mouse models (57). Post-transfection, cells were treated with vehicle (0.1% ethanol) or physiological levels of androgen (0.1 nM DHT) for 20–24 h. Cells were then harvested and processed to detect luciferase and β -galactosidase activity. As can be seen in Fig. 1B, the probasin promoter was induced 138.0-fold by androgen in CV1 cells (left panel) but only 6.6- and 10.0-fold in SWI/SNF-defective SW13 and C33A cells, respectively (middle and right panels). This significant reduction in activity is comparable to that observed with GR in C33A and SW13 cells (29). Thus, the AR exhibits significant deficiency in activating the probasin promoter in the absence of core SWI/SNF activity.

AR-dependent Activation of the Probasin Promoter Is Induced by the Restoration of BRG1 but Preferentially by hBRM—It has been previously shown that SWI/SNF activity can be restored in SW13 and C33A cells through ectopic expression of BRG1 or hBRM. For example, CD44 expression is lost in BRG1/hBRM-deficient cell lines. The expression of CD44 expression can be restored by transfecting either BRG1 or hBRM into these cells (SW13 or C33A) (44, 45, 48). In addition,

FIG. 2. AR-dependent activation of the probasin promoter is induced by the restoration of BRG1, but preferentially by hBRM. *A*, C33A cells were transfected with expression plasmid for empty vector (pcDNA3.1(+)) (lane 1), BRG1 (lane 2), or hBRM (lane 3). Thirty-six hours post-transfection, cells were harvested, lysed, and subjected to SDS-PAGE. BRG1, hBRM, and CD44 levels were detected by immunoblot. Immunoblots for β -tubulin were included as a loading control. *B*, to determine the impact of SWI/SNF on probasin activation, SW13 cells were transfected as in Fig. 1 with the plasmids and amounts (in micrograms), as indicated. The cells were then washed, allowed to recover for 4–6 h, and stimulated with either vehicle (0.1% ethanol) or 0.1 nM dihydrotestosterone (DHT), as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase (β -gal) activities. Basal AR activity was set to “1,” and relative luciferase activity is shown. *C*, to examine the relative efficacy of BRG1 versus hBRM for ER activation, SW13 cells were transfected as in Fig. 1 with the plasmids and amounts (in micrograms), as indicated. Cells were then washed, allowed to recover for 4–6 h and stimulated with either vehicle (0.1% ethanol) or 10 nM 17 β -estradiol (E2), as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase (β -gal) activities. Basal ER activity was set to “1,” and relative luciferase activity is shown.

we and others have previously shown that BRG1 or hBRM is required for RB-mediated repression of the cyclin A promoter. Re-introduction of BRG1 or hBRM into a SWI/SNF-deficient background restores the ability of RB to repress cyclin A (as observed both through reporter assay and through examination of endogenous protein) (46, 50). Thus, restoration of SWI/SNF activity can rescue transcriptional defects from both ectopic reporter constructs and endogenous promoters. As shown in Fig. 2A, the function of the BRG1 and hBRM expression

constructs were confirmed by transfecting the expression plasmids into C33A cells and immunoblotting for CD44 expression (compare lane 1 to lanes 2 and 3). As can be seen, both BRG1 and hBRM were effective at restoration of CD44 expression. hBRM was more efficient in the induction of CD44, which is consistent with previous reports (48). BRG1 and hBRM expression levels are shown, and immunoblots against β -tubulin were included as a loading control. To determine if restoration of SWI/SNF ATPase activity could induce transcription of the probasin promoter, reporter assays were repeated in the presence of ectopic BRG1 or hBRM. Briefly, SW13 cells were cultured, as in Fig. 1, in the absence of steroid hormone and transfected with plasmid encoding AR, BRG1 or hBRM, β -galactosidase, and the ARR2-luc reporter at the amounts indicated. Post-transfection, cells were stimulated with either DHT or ethanol vehicle. BRG1 or hBRM expression had no effect on basal AR activity (in the presence of ethanol) (Fig. 2B). In the presence of DHT, BRG1 marginally stimulated ligand-dependent promoter activation (2.5-fold, as compared with the absence of BRG1). In comparison, hBRM markedly stimulated AR activity (8.4-fold, compared with the absence of hBRM or BRG1). Thus, AR exhibited a preference for hBRM as the SWI/SNF ATPase component for transcriptional activation of the ARR2-luc reporter. To demonstrate that these effects were attributed to ATPase activity and not to simple assembly of the SWI/SNF complex, dominant negative BRG1 (dnBRG1) was utilized. This allele harbors point mutations in the ATPase domain, rendering the resulting mutant protein capable of assembling the SWI/SNF complex, but incapable of remodeling chromatin (56). As can be seen, dnBRG1 lacked any ability to restore AR activity on the probasin promoter (Fig. 2B).

Given the preference of AR for hBRM containing SWI/SNF activity, we examined the effect of BRG1 versus hBRM on ER α activity. It has been shown that the re-introduction of BRG1 into SW13 cells restores ER-mediated activation of the 3X-ERE-luc reporter and that dnBRG1 does not allow for the activation of the same reporter (34). These data were confirmed in Fig. 2C, wherein SW13 cells were transfected with expression plasmid for BRG1 or dnBRG1 and the ERE-luc reporter. Previous reports did not examine the relative efficacy of hBRM for restoration of ER activity. As depicted in Fig. 2C, ER α activity was equally restored by BRG1 versus hBRM in SW13 cells (3.3- and 3.6-fold above background for BRG1 and hBRM, respectively). These collective data imply that AR has a preference for hBRM as the core ATPase, whereas BRG1 and hBRM are equally effective in stimulating ER α activity.

Activation of the Proximal PSA Promoter Requires hBRM-dependent SWI/SNF Activity—Given the unique preference of AR for hBRM containing SWI/SNF complexes, these results prompted examination of additional AR-dependent promoter constructs. The prostate-specific antigen (PSA) promoter is the most well characterized AR target, is tightly regulated by androgens, and demonstrates prostate-specific expression (55). The proximal PSA promoter contains two ARE sites and was cloned upstream of luciferase to generate the PSA-luc reporter. As can be seen in Fig. 3A, AR activated the PSA promoter ~10.1-fold in the presence of ligand in CV1 cells (*left panel*). In contrast, no activation of PSA-luc was observed in SW13 cells (*right panel*). To analyze the effect of SWI/SNF activity on activation of this promoter, BRG1 or hBRM were restored by transfection, as seen in Fig. 2B. Strikingly, restoration of BRG1 failed to rescue PSA-luc activation, whereas hBRM strongly induced promoter activity, yielding a 278% increase over activity in the presence of ligand but absence of hBRM (Fig. 3B). Thus, activation of both the proximal probasin promoter and the proximal PSA promoter requires SWI/SNF activity, with a

FIG. 3. Activation of the proximal PSA promoter requires hBRM-dependent SWI/SNF activity. A, CV1 (left panel) and SW13 (right panel) cells were seeded in steroid-free media and transfected with expression plasmid for AR and the PSA-luc reporter, as described under "Experimental Procedures." The cells were then washed, allowed to recover for 4–6 h, and stimulated with either vehicle (0.1% ethanol) or 0.1 nM dihydrotestosterone (DHT), as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase activities. Basal AR activity was set to "1," and relative luciferase activity is shown. B, to determine the impact of SWI/SNF on PSA activation, SW13 cells were transfected as in Fig. 2B with the plasmids and amounts (in micrograms), as indicated. The cells were then washed, allowed to recover for 4–6 h, and stimulated with either vehicle (0.1% ethanol) or 0.1 nM DHT, as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase activities. Basal AR activity was set to "1," and relative luciferase activity is shown. C, to determine the effect of excess hBRM on probasin activation in a SWI/SNF competent cell line, CV1 cells were transfected as in Fig. 3B with the plasmids and amounts (in micrograms) as indicated. The cells were then washed, allowed to recover for 4–6 h, and stimulated with either vehicle (0.1% ethanol) or 0.1 nM DHT, as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase activities. Basal AR activity was set to "1," and relative luciferase activity is shown.

strong preference for hBRM as the core ATPase. Interestingly, addition of hBRM in SWI/SNF-competent CV1 cells had no impact on the proximal PSA promoter (Fig. 3C), or the proximal probasin promoter (data not shown), indicating that excess hBRM does not simply enhance AR activity.

The PSA Enhancer Region Facilitates a Bypass of the SWI/SNF Requirement—The preceding data revealed a requirement for SWI/SNF activity on the PSA promoter. For this target, a distal regulatory element has been characterized upstream of the proximal promoter. This enhancer region is known to harbor a site of AR binding (ARE III, ~4 kb upstream) and to synergize with the proximal PSA promoter for androgen-dependent gene activation. To analyze the impact of the enhancer region on SWI/SNF activity, the previously characterized PSA61-luc construct was utilized (55). As shown in Fig. 4A, this construct contains both the proximal promoter and the upstream enhancer region (–6000 to +12). Similar to Fig. 1, SWI/SNF-defective (SW13 and C33A) and competent (CV1) cells were transfected in the absence of steroid hormone with the expression plasmid for AR, β -galactosidase, and the PSA reporter construct (PSA61-luc). Strikingly, the ability of AR to activate the PSA61-luc construct was comparable in each cell line tested (Fig. 4B), with the highest activation above basal levels (13.0-fold) being observed in C33A cells (3.8-fold in SW13

and 5.7-fold in CV1). Thus, the presence of the enhancer region in PSA facilitates a bypass of the SWI/SNF requirement.

To determine if re-introduction or overexpression of hBRM could further augment transcription of the PSA61-luc reporter, SW13 and CV1 cells were transfected as above with expression plasmid for hBRM at the concentrations indicated, and stimulated with either vehicle or DHT post-transfection. As shown, hBRM did not significantly increase the transcriptional levels of the PSA61-luc reporter in either cell background (Fig. 4, C and D). BRG1 was also incapable of potentiating the response to ligand (data not shown). Lastly, re-introduction of BRG1 or hBRM into androgen-dependent LNCaP cells did not significantly increase expression of the endogenous PSA gene (data not shown). Therefore, not only does the enhancer region effectively reverse the requirement for SWI/SNF activity but it renders the proximal PSA promoter refractory to SWI/SNF action.

To examine the effect of the enhancer on the proximal probasin promoter, the ARR2+E-luc construct was generated, which positions the enhancer region of PSA (containing the ARE III) directly upstream of the ARR2 promoter (Fig. 5A) (55, 57). Transfection of this reporter construct into SW13 or C33A cells with AR expression plasmid, as described for Fig. 1, revealed that, unlike the PSA61 reporter, activity of the enhancer

FIG. 4. The PSA enhancer region facilitates a bypass of the SWI/SNF requirement. A, diagram of the PSA-luc and PSA61-luc constructs. PSA-luc contains only the core PSA promoter, whereas PSA61-luc includes a distal upstream enhancer element. B, similar to Fig. 3A, CV1 (left), SW13 (middle), or C33A (right) cells were transfected with plasmid encoding AR and PSA61-luc and treated with either 0.1% ethanol or 0.1 nM DHT, as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase activities. Basal AR activity was set to “1,” and relative luciferase activity is shown. C and D, to determine the effect of excess hBRM on SWI/SNF refractory enhancer/promoter activity, CV1 (C) or SW13 (D) cells were transfected with the indicated expression plasmids, treated and analyzed as in part B. Basal AR activity was set to “1,” and relative luciferase activity is shown.

addition to the probasin promoter did not alter the response to SWI/SNF activity (Fig. 5, B and C). Ligand-dependent activation of the reporter constructs was similar between ARR2-LUC and ARR2+E-LUC (6.6- versus 6.5-fold, respectively). As with the proximal promoter, activity of ARR2+E was restored upon co-transfection of hBRM (55.0- versus 42.0-fold, respectively). Thus, the impact of the enhancer on SWI/SNF requirement is variant and dependent on promoter context.

DISCUSSION

In this report, we show that AR exhibits a differential requirement for SWI/SNF activity. The core PSA promoter demonstrated a requirement for SWI/SNF activity, whereas the probasin promoter retained weak activity in the absence of SWI/SNF. Androgen-dependent activation of the probasin promoter could be partially enhanced with BRG1, but the PSA promoter could not be restored by BRG1. Both of these promot-

ers were strongly activated by AR in the presence of hBRM. Thus, AR exhibits a preference for hBRM containing SWI/SNF activity. This is distinct from what we observed with the ER α , wherein BRG1 and hBRM demonstrated equal efficacy in the ability to restore ER α transactivation potential. In addition, we show that the distal PSA regulatory element strongly influences the requirement of hBRM activity for AR-dependent transcription on the PSA promoter. The presence of the upstream enhancer region bypassed the requirement for SWI/SNF and rendered the element refractory to excess SWI/SNF activity. In contrast, addition of this same enhancer element to the proximal probasin promoter did not alter the requirement for the SWI/SNF complex. Together, these data indicate that the requirement of AR for SWI/SNF activity is dependent on promoter context and is more receptive to SWI/SNF complexes with hBRM as the core ATPase.

FIG. 5. The PSA enhancer does not circumvent the requirement of SWI/SNF activity on the probasin promoter. A, diagram of ARR2-luc and ARR2+E-luc plasmid constructs. ARR2-luc contains both probasin ARE regions in tandem, as indicated. ARR2+E-luc is chimeric construct that contains the PSA enhancer region. B and C, to determine if the PSA enhancer can also confer SWI/SNF independence to the core probasin promoter, SW13 (B) or C33A (C) cells were transfected, as described in Fig. 4, with the plasmids and quantities indicated. The cells were then washed, allowed to recover for 4–6 h, and stimulated with either vehicle (0.1% ethanol) or 0.1 nM DHT, as indicated. 20–24 h post-stimulation, cells were harvested, lysed, and monitored for luciferase and β -galactosidase activities. Basal AR activity was set to “1,” and relative luciferase activity is shown.

SWI/SNF activity has been implicated in the appropriate regulation of specific genes. Here, we show that the PSA promoter shows a distinct requirement for SWI/SNF. Probasin, however, retains some activity in the absence of SWI/SNF (6-fold induction). The disparity in requirement for SWI/SNF activity between the two core promoters indicates that the probasin promoter does not explicitly require chromatin remodeling for responsiveness, suggesting that AR likely binds to the probasin AREs in the absence of these factors. However, the probasin promoter was still receptive to SWI/SNF activity (especially hBRM), suggesting that remodeling may assist in a secondary step of activation (*e.g.* promoter clearance). In contrast, the PSA promoter may require remodeling activity at an earlier step of transcriptional activation (*e.g.* AR access). It is interesting to note that basal PSA activity (in the absence of ligand) is significantly reduced upon the re-introduction of BRG1 or hBRM (Fig. 3, B and C), whereas no effect on basal activity was observed with the probasin promoter. Because remodeling proteins can influence several classes of transcription factors (including the loading of both activators and repressors), it is possible that these observations reflect the loading of repressors to the promoter in the absence of hormone.

Strikingly, the presence of the distal PSA enhancer region alleviated the SWI/SNF requirement for the PSA promoter. This region has been well characterized and is known to contain AR binding sites (*e.g.* ARE III) (55). Recently, it has been

shown through chromatin immunoprecipitation experiments that ARE III is a key site of AR interaction. Using quantitative PCR to analyze chromatin immunoprecipitation data, a recent report revealed that AR binds first to ARE III, preceding histone acetylation on this region, whereas AR binding to the promoter region (ARE I) was delayed and occurred after histone acetylation in that region (17). Thus, the distal enhancer region may be the initial site of AR action, capable of influencing events at the proximal promoter. A contrasting earlier report demonstrated that AR binding to ARE III and the ARE I region was simultaneous but that co-repressors were recruited only to the proximal promoter (60). In either scenario, co-activators known to bind the ARE III region may alleviate the requirement of SWI/SNF function in the proximal promoter. The data contained herein suggest that these events do not require SWI/SNF but that ablation of enhancer activity results in a SWI/SNF-dependent proximal promoter. Interestingly, addition of the PSA enhancer region immediately 5' to the proximal probasin promoter did not relieve SWI/SNF dependence, indicating that structural or positional effects of this region (especially spacing between cis-acting elements) could influence a bypass of the SWI/SNF requirement. As of yet, there is scant knowledge of how the enhancer and proximal PSA promoter (a 4-kb separation) interact to regulate AR-dependent transcription. Although the enhancer does contain

core known to contain at least three separate regions of activity required for its function (55). Thus, it is evident that multiple cis-acting elements contribute to ARE III function in the enhancer region. The role of the enhancer in controlling the influence of the SWI/SNF complex will be the focus of future study.

No studies have investigated the relative contribution of BRG1 *versus* hBRM complexes for nuclear receptor activation. Although both BRG1 and hBRM have been shown to associate with the GR, no comparison of preference for the ATPases has been performed (29–31). Here, we report that AR function on multiple target promoters shows a strong preference for hBRM activity, whereas ER α activity was effectively restored by either BRG1 or hBRM. Enhancement of AR activity by hBRM on a nonspecific steroid hormone reporter (murine mammary tumor virus) was also recently reported to occur in SWI/SNF-competent cells, which is consistent with our findings (61). Preference for a core ATPase does have precedent in the literature. For example, two ankyrin repeat proteins integral to the Notch pathway selectively associate with BRM and not BRG1. In contrast, several zinc finger proteins were recently shown to associate with and be activated by BRG1, but not BRM, through virtue of unique N-terminal sequences present only on BRG1. In that study, interaction with BRG1 was shown to occur through the zinc finger protein DNA binding domain, including that of the retinoic acid receptors (RXR and RAR) (62). It is interesting to note that both AR and ER α contain a highly conserved central DNA binding domain comprised of two zinc finger-like modules, yet neither shows a preference for BRG1-containing complexes (63, 64). Thus, divergent N-terminal sequence in the nuclear receptors may influence activation by BRG1- *versus* hBRM-containing complexes.

The biological outcome of hBRM or BRG1 preference is unclear. Studies with knockout mice have demonstrated that ablation of BRG1 expression is lethal in the developing embryo but is not required for viability in primary embryonic fibroblasts, as determined via Cre-lox technology (65). Thus, BRG1 is specifically required for development. In contrast, BRM knockout mice are viable, indicating that BRM function is dispensable for development (66). In differentiating cells, BRM is expressed at high levels. Interestingly, loss of BRM also results in a small decrease in the size of the testis (an androgen-dependent tissue) (67) as compared with wild-type controls (66). The impact of hBRM loss on the growth of the prostate has yet to be determined. Relative expression of BRG1 and hBRM in prostatic adenocarcinomas has not been addressed, although we have detected both proteins in LNCaP (AR-positive, androgen-dependent human prostatic adenocarcinoma) cells (data not shown). The BRG1 locus is reported to be mutated in AR-negative, androgen-independent DU145 (prostate cancer) cells, resulting in a frameshift prior to the ATPase domain and likely encoding a non-functional protein (68). The impact of this mutation on cellular function is unknown, although DU145 cells are known to support AR activity upon re-introduction of the protein (69), thus supporting our observations that hBRM is the preferred ATPase for AR action. In addition, the BRG1 locus (19p13) is a known region of loss of heterozygosity in prostate cancer, and a re-introduction of 19p13.1–13.2 in the Dunning rat R3327 prostate cancer model suppresses tumor growth (70). This function of BRG1 may be attributed to the requirement of RB for SWI/SNF function, and the effect of BRG1 loss on androgen receptor activity in prostate tumor cells is unknown. Examination of hBRM expression in prostate cancer progression has not been addressed. However, loss of hBRM expression is known to result in induction of BRG1, indicating that compensation between core ATPases takes place (66).

Should this be the case, our data would suggest that loss of BRG1 and concomitant induction of hBRM could serve to enhance AR action in the prostate.

In summary, we report a functional interaction between AR and hBRM-containing SWI/SNF complexes and demonstrate that different AR targets show disparity in the requirement for SWI/SNF. Our data also indicate that access to enhancer regions may influence target specificity for SWI/SNF function. These findings provide the first link between AR and hBRM function and provide the impetus to study SWI/SNF impact on multiple AR targets and regulatory elements.

Acknowledgments—We thank Craig Burd, Janet Hess-Wilson, Steve Angus, and Drs. B. Weissman and E. Knudsen for advice and critical reading of the manuscript. We also thank both Knudsen laboratories for advice and aiding us in our efforts.

REFERENCES

- Feldman, B. J., and Feldman, D. (2001) *Nat. Rev. Cancer* **1**, 34–45
- Montgomery, J. S., Price, D. K., and Figg, W. D. (2001) *J. Pathol.* **195**, 138–146
- Culig, Z., Klocker, H., Bartsch, G., and Hobisch, A. (2002) *Endocr. Relat. Cancer* **9**, 155–170
- Brinkmann, A. O., and Trapman, J. (2000) *Adv. Pharmacol.* **47**, 317–341
- Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990) *Endocr. Rev.* **11**, 201–220
- Gelmann, E. P. (2002) *J. Clin. Oncol.* **20**, 3001–3015
- Stearns, M. E., and McGarvey, T. (1992) *Lab. Invest.* **67**, 540–552
- Santen, R. J. (1992) *J. Clin. Endocrinol. Metab.* **75**, 685–689
- Denis, L. J., and Griffiths, K. (2000) *Semin. Surg. Oncol.* **18**, 52–74
- Pirtskhalaishvili, G., Hrebinko, R. L., and Nelson, J. B. (2001) *Cancer Pract.* **9**, 295–306
- Klotz, L. (2000) *Cancer* **88**, 3009–3014
- Catalona, W. J., Ramos, C. G., and Carvalhal, G. F. (1999) *CA Cancer J. Clin.* **49**, 282–296
- Masiello, D., Cheng, S., Bubley, G. J., Lu, M. L., and Balk, S. P. (2002) *J. Biol. Chem.* **277**, 26321–26326
- Grossmann, M. E., Huang, H., and Tindall, D. J. (2001) *J. Natl. Cancer Inst.* **93**, 1687–1697
- Cude, K. J., Dixon, S. C., Guo, Y., Lisella, J., and Figg, W. D. (1999) *J. Mol. Med.* **77**, 419–426
- Balk, S. P. (2002) *Urology* **60**, 132–138; discussion, 138–139
- Jia, L., Kim, J., Shen, H., Clark, P. E., Tilley, W. D., and Coetzee, G. A. (2003) *Mol. Cancer Res.* **1**, 385–392
- Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S., and Wilson, E. M. (2001) *Cancer Res.* **61**, 4315–4319
- Heinlein, C. A., and Chang, C. (2002) *Endocr. Rev.* **23**, 175–200
- Sommer, S., and Fuqua, S. A. (2001) *Semin. Cancer Biol.* **11**, 339–352
- McKenna, N. J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 11697–11702
- Collingwood, T. N., Urnov, F. D., and Wolffe, A. P. (1999) *J. Mol. Endocrinol.* **23**, 255–275
- Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev.* **14**, 121–141
- Young, J. C., and Hartl, F. U. (2002) *Nat. Struct. Biol.* **9**, 640–642
- Yeh, S., and Chang, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5517–5521
- Zhou, Z. X., He, B., Hall, S. H., Wilson, E. M., and French, F. S. (2002) *Mol. Endocrinol.* **16**, 287–300
- Wang, Q., Sharma, D., Ren, Y., and Fondell, J. D. (2002) *J. Biol. Chem.* **277**, 42852–42858
- Yoshinaga, S. K., Peterson, C. L., Herskowitz, I., and Yamamoto, K. R. (1992) *Science* **258**, 1598–1604
- Muchardt, C., and Yaniv, M. (1993) *EMBO J.* **12**, 4279–4290
- Fletcher, T. M., Xiao, N., Mautino, G., Baumann, C. T., Wolford, R., Warren, B. S., and Hager, G. L. (2002) *Mol. Cell. Biol.* **22**, 3255–3263
- Fryer, C. J., and Archer, T. K. (1998) *Nature* **393**, 88–91
- Chiba, H., Muramatsu, M., Nomoto, A., and Kato, H. (1994) *Nucleic Acids Res.* **22**, 1815–1820
- Ichinose, H., Garnier, J. M., Chambon, P., and Losson, R. (1997) *Gene (Amst.)* **188**, 95–100
- DiRenzo, J., Shang, Y., Phelan, M., Sif, S., Myers, M., Kingston, R., and Brown, M. (2000) *Mol. Cell Biol.* **20**, 7541–7549
- Belandia, B., Orford, R. L., Hurst, H. C., and Parker, M. G. (2002) *EMBO J.* **21**, 4094–4103
- Wang, W., Xue, Y., Zhou, S., Kuo, A., Cairns, B. R., and Crabtree, G. R. (1996) *Genes Dev.* **10**, 2117–2130
- Peterson, C. L., and Workman, J. L. (2000) *Curr. Opin. Genet. Dev.* **10**, 187–192
- Tyler, J. K., and Kadonaga, J. T. (1999) *Cell* **99**, 443–446
- Klochendler-Yeivin, A., Muchardt, C., and Yaniv, M. (2002) *Curr. Opin. Genet. Dev.* **12**, 73–79
- Muchardt, C., and Yaniv, M. (2001) *Oncogene* **20**, 3067–3075
- Muchardt, C., and Yaniv, M. (1999) *J. Mol. Biol.* **293**, 187–198
- King, I. F., and Kingston, R. E. (2001) *Nature* **414**, 858–861
- Narlikar, G. J., Fan, H. Y., and Kingston, R. E. (2002) *Cell* **108**, 475–487
- Strobeck, M. W., DeCristofaro, M. F., Banine, F., Weissman, B. E., Sherman, L. S., and Knudsen, E. S. (2001) *J. Biol. Chem.* **276**, 9273–9278
- Strobeck, M. W., Reisman, D. N., Gunawardena, R. W., Betz, B. L., Angus, S. P., Knudsen, K. E., Kowalik, T. F., Weissman, B. E., and Knudsen, E. S. (2002) *J. Biol. Chem.* **277**, 4782–4789

46. Strobeck, M. W., Knudsen, K. E., Fribourg, A. F., DeCristofaro, M. F., Weissman, B. E., Imbalzano, A. N., and Knudsen, E. S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7748–7753
47. Dunaief, J. L., Strober, B. E., Guha, S., Khavari, P. A., Alin, K., Luban, J., Begemann, M., Crabtree, G. R., and Goff, S. P. (1994) *Cell* **79**, 119–130
48. Reisman, D. N., Strobeck, M. W., Betz, B. L., Sciarriotta, J., Funkhouser, W. Jr., Murchardt, C., Yaniv, M., Sherman, L. S., Knudsen, E. S., and Weissman, B. E. (2002) *Oncogene* **21**, 1196–1207
49. Dahiya, A., Gavin, M. R., Luo, R. X., and Dean, D. C. (2000) *Mol. Cell. Biol.* **20**, 6799–6805
50. Zhang, H. S., Postigo, A. A., and Dean, D. C. (1999) *Cell* **97**, 53–61
51. Bourachot, B., Yaniv, M., and Muchardt, C. (1999) *Mol. Cell. Biol.* **19**, 3931–3939
52. Singh, P., Coe, J., and Hong, W. (1995) *Nature* **374**, 562–565
53. Singh, P., Chan, S. W., and Hong, W. (2001) *J. Biol. Chem.* **276**, 13762–13770
54. Wallberg, A. E., Neely, K. E., Hassan, A. H., Gustafsson, J. A., Workman, J. L., and Wright, A. P. (2000) *Mol. Cell. Biol.* **20**, 2004–2013
55. Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W., and Trapman, J. (1997) *Mol. Endocrinol.* **11**, 148–161
56. Khavari, P. A., Peterson, C. L., Tamkun, J. W., Mendel, D. B., and Crabtree, G. R. (1993) *Nature* **366**, 170–174
57. Zhang, J., Thomas, T. Z., Kasper, S., and Matusik, R. J. (2000) *Endocrinology* **141**, 4698–4710
58. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752
59. Decristofaro, M. F., Betz, B. L., Rorie, C. J., Reisman, D. N., Wang, W., and Weissman, B. E. (2001) *J. Cell. Physiol.* **186**, 136–145
60. Shang, Y., Myers, M., and Brown, M. (2002) *Mol. Cell* **9**, 601–610
61. Inoue, H., Furukawa, T., Giannakopoulos, S., Zhou, S., King, D. S., and Tanese, N. (2002) *J. Biol. Chem.* **277**, 41674–41685
62. Kadam, S., and Emerson, B. M. (2003) *Mol. Cell* **11**, 377–389
63. Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., and Wilson, E. M. (1988) *Science* **240**, 327–330
64. Evans, R. M., and Hollenberg, S. M. (1988) *Cell* **52**, 1–3
65. Bultman, S., Gebuhr, T., Yee, D., La Mantia, C., Nicholson, J., Gilliam, A., Randazzo, F., Metzger, D., Chambon, P., Crabtree, G., and Magnuson, T. (2000) *Mol. Cell* **6**, 1287–1295
66. Reyes, J. C., Barra, J., Muchardt, C., Camus, A., Babinet, C., and Yaniv, M. (1998) *EMBO J.* **17**, 6979–6991
67. Desjardins, C. (1978) *J. Anim. Sci.* **47**, Suppl. 2, 56–79
68. Wong, A. K., Shanahan, F., Chen, Y., Lian, L., Ha, P., Hendricks, K., Ghaffari, S., Iliev, D., Penn, B., Woodland, A. M., Smith, R., Salada, G., Carillo, A., Laity, K., Gupte, J., Swedlund, B., Tavtigian, S. V., Teng, D. H., and Lees, E. (2000) *Cancer Res.* **60**, 6171–6177
69. Miyamoto, H., Rahman, M., Takatera, H., Kang, H. Y., Yeh, S., Chang, H. C., Nishimura, K., Fujimoto, N., and Chang, C. (2002) *J. Biol. Chem.* **277**, 4609–4617
70. Gao, A. C., Lou, W., Ichikawa, T., Denmeade, S. R., Barrett, J. C., and Isaacs, J. T. (1999) *Prostate* **38**, 46–54

Chapter III

BAF57 Governs Androgen Receptor Action and Androgen-Dependent Proliferation through SWI/SNF

Kevin A. Link,¹ Craig J. Burd,¹ Erin Williams,¹ Thomas Marshall,¹ Gary Rosson,³ Erin Henry,³ Bernard Weissman,³ and Karen E. Knudsen^{1, 2*}

¹Department of Cell Biology, and ²Center for Environmental Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0521, USA. ³The Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7295

*Corresponding Author. Mailing address: Department of Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521. Phone: (513) 558-7371. Fax: (513) 558-4454. E-mail: karen.knudsen@uc.edu

ABSTRACT

Androgen receptor (AR) activity is required for prostate cancer development and progression. Thus, there is a major impetus to understand the regulation of AR action. We and others have previously shown that AR transactivation potential is dependent on the presence of an active SWI/SNF chromatin remodeling complex. However, the mechanisms underlying SWI/SNF regulation of the AR remained unsolved. Here, we show that the BAF57 subunit, an accessory component of the remodeling complex, is a critical regulator of AR function. We show that BAF57 is expressed in the luminal epithelia of the prostate, and is required for AR-dependent transactivation in prostatic adenocarcinoma cells. Our data reveal that BAF57 can directly bind to the AR and is recruited to endogenous AR targets upon ligand activation. Loss of BAF57 or abrogation of BAF57 function severely compromised AR activity, as observed using both exogenous and endogenous AR targets. Rescue of BAF57 function restored AR activity, thus demonstrating a specific requirement of BAF57 for AR activity. This action of BAF57 proved to be dependent on SWI/SNF ATPase function. BAF57 has previously been implicated in nuclear receptor co-activator function, and we show that while BAF57 facilitated co-activator activity, only a selected subset required BAF57 for co-activator function. Lastly, we demonstrate that both BAF57 and BRM are required for proliferation of AR-dependent prostatic adenocarcinoma cells. In summary, these findings identify BAF57 as a critical modulator of the AR, capable of altering AR activity, co-activator function, and AR dependent proliferation.

INTRODUCTION

The androgen receptor (AR) is a member of the nuclear receptor superfamily, whose activity is required for prostate development, growth, and survival (16, 26, 34). This dependence on AR function is exploited in prostate cancer therapy, wherein AR remains the major target for therapeutic intervention (1, 34). Given the importance of AR in treatment of this tumor type, considerable effort has been directed at revealing the mechanisms of AR action.

The AR is a member of the nuclear receptor superfamily of transcription factors, and is activated by ligand binding to induce transactivation potential. Testosterone, the most prevalent AR ligand in the sera, is converted to dihydrotestosterone (DHT) in the prostate through the action of 5- α reductase. DHT serves as a high affinity ligand for the AR, and stimulates a cascade of events which initiate AR activation (6, 16, 52). Initially, AR is released from heat shock proteins, undergoes homodimerization, and rapidly translocates to the nucleus where it is targeted to androgen response elements (AREs) located within AR target gene promoters/enhancers. Once activated by ligand, AR recruits a host of cofactors that facilitate the formation of an active transcriptional complex (23, 59). In general, these cofactors act to release DNA from chromatin through either covalent modification of histones or remodeling of chromatin structure. Such modifications foster a state of open chromatin, allowing recruitment of basal transcription machinery and RNA polymerase II (27, 46, 62, 68).

Several classes of AR co-activators promote histone modifications. Members of the p160 co-activator family (including SRC1, SRC2/TIF2/GRIP1, and SRC3/RAC3/AIB1) serve as platforms for the recruitment of histone acetyltransferases (HATs), such as CBP/p300 or p/CAF. SRC1 and SRC3 also harbor intrinsic HAT activity, which likely contributes to their action as co-activators (12, 63, 67). Acetylation of core histones by the recruited HATs results in a displacement or loosening of DNA from the nucleosome, thus facilitating the recruitment of the subsequent co-factors and the basal transcription machinery. In addition, P/CAF has been shown to acetylate the AR itself, an event shown to be essential for AR activity (20). Thus,

through their ability to modify both AR and core histones, the p160 co-activators play pivotal roles in AR activation. The importance of such co-activator regulation in prostate cancer is apparent, as aberrant expression of both SRC1 and TIF2 is associated with relapse after therapeutic intervention (21, 24). Many additional AR co-activators have been identified whose actions are less well described. For example, ARA70 and ARA55, in addition to enhancing AR activity, are able to increase the androgenic activity of anti-androgens such as hydroxyflutamide (22, 44). Recently, ARA70 expression was shown to be enhanced in the neoplastic prostate compared to benign tissue, thus further implicating a role for this co-activator in prostate cancer progression (29). Lastly, the E2 ubiquitin-conjugating enzyme, Ubc9, has been shown to bind directly to and activate the AR, independent of its SUMO-1 conjugation function (51). The coordinate actions of these co-activators are thought to play pivotal roles in AR activation, and likely influence tissue specific AR activity. The contribution of these co-activators with regard to histone acetylation and cooperation with the p160 family has yet to be thoroughly examined.

Although the p160 co-activators initiate histone modifications through acetylation, it has been shown that several nuclear receptors also attract complexes that enzymatically regulate chromatin structure. Several classes of chromatin remodeling complexes have been described, including WINAC, NuRD, ISWI, and SWI/SNF (4, 39, 46, 70, 72). ARIP4 has been described as a SWI/SNF like chromatin remodeling enzyme, and was shown to modulate AR activity (57, 61). In general, chromatin remodeling complexes utilize the hydrolysis of ATP to disrupt histone-DNA association, thus altering chromatin structure to foster a state of either closed chromatin (resulting in gene repression) or open chromatin (resulting in enhanced gene transcription). Although the precise mechanisms of SWI/SNF action *in vivo* have yet to be elucidated, associated activities *in vitro* include alterations in nucleosome structure, nucleosome sliding, dinucleosome formation, and histone octamer transfer (17, 46). Such alterations in chromatin structure can be distinguished from histone modifications, and are not dependent on histone acetylation (30). Moreover, SWI/SNF action has been shown to contribute to the dynamic

activity of nuclear receptors, facilitating rapid mobilization of the receptors following SWI/SNF recruitment (18). The activity of several nuclear receptors, including the estrogen receptor (ER) and glucocorticoid receptor (GR), has been shown to be enhanced by the action of SWI/SNF (3, 10, 14, 18, 19, 31, 45, 50, 72, 74). SWI/SNF represents a class of large, 2 MDa chromatin remodeling complexes which contain a central ATPase (either BRM/SNF2alpha or BRG1/SNF2beta) and a variable series of associated subunits termed BRG1-associated factors (BAFs). SWI/SNF exists as a multitude of biochemically diverse complexes, and although the function of each specialized complex remains unclear, it has been speculated that variances in combinatorial assembly dictate diverse biological outcomes. Supporting this view, it has been shown that BRG1 and BRM demonstrate specificity for protein-protein interaction and likely govern unique gene targets (35). Although some redundancies do exist, BRG1 is predominantly found in cell types undergoing proliferation or self-renewal, whereas BRM expression is increased after cellular differentiation. Interestingly, BRM was recently shown to be expressed in normal human prostatic epithelia, whereas BRG1 could not be detected in this tissue (53). We and others have shown that the AR requires SWI/SNF function for activity, and our studies highlighted a specific role for BRM complexes in AR regulation (30, 32, 43). However, the mechanism by which BRM specifically regulates AR activity remained unsolved.

It is speculated that the BAF subunits largely contribute to SWI/SNF specificity (47). Subunits commonly associated with the SWI/SNF complex include BAF155, BAF170, INI1/SNF5, BAF250, BAF60, and BAF57 (46, 71, 72). Several of these subunits have been shown to harbor critical functions. For example, the loss of the INI1/SNF5 subunit gives rise to rhabdoid tumors in humans and can promote tumorigenesis, thus identifying INI1/SNF5 as a tumor suppressor (69). Subsequent studies demonstrated that reintroduction of INI1/SNF5 into human tumor cell lines deficient in this subunit induced cell cycle arrest (5). Other subunits, including BAF250, BAF60a, and BAF57 have been shown to directly interact with GR, and to promote GR activation (28, 47). Lastly, the BAF57 subunit has been shown to interact with both

the ER and the p160 co-activators. Although BAF57 function was dispensable for ER activation, BAF57 was essential for SRC1 to modulate the ER (3). Collectively, these observations implicate SWI/SNF subunits as critical regulators of nuclear receptor-SWI/SNF interactions.

BAF57 itself contains DNA binding capability through its HMG (high mobility group) motif and a kinesin-like coiled-coil domain, and is suggested to play an architectural role in the formation of higher order protein complexes. Although these properties of BAF57 are dispensable for SWI/SNF chromatin remodeling activity (71), it is hypothesized that BAF57 may lend specificity in targeting to the SWI/SNF complex. This contention was supported by observations in transgenic mice, wherein mutations in the HMG domain of BAF57 ablated specific SWI/SNF target gene regulation (9).

Here, we show that BAF57 is expressed in the AR-positive luminal epithelial layer of the prostate, and that BAF57 strongly regulates AR function. We show that BAF57 directly binds to AR, and is recruited to AR regulatory regions in a ligand-dependent fashion. Using both exogenous and endogenous prostate-specific AR targets, we show that AR requires BAF57 function for robust transcriptional response. We also demonstrate that this function of BAF57 requires SWI/SNF ATPase function, firmly linking BAF57 action to alterations in nucleosome structure. Similar to what was observed with ER, BAF57 also facilitated p160-mediated AR enhancement, and a similar profile was observed with Ubc9. Interestingly, ARA70 and ARA55 maintained activity in the absence of BAF57, but were augmented by BAF57 for AR activation. Thus, BAF57 exhibited co-activator specific modulation of AR activity. Given the observed necessity of BAF57 for AR target gene transcription and co-activator function, we examined the requirement of BAF57 and SWI/SNF for proliferation in AR dependent prostate cancer cell growth. Strikingly, abrogation of either BAF57 or BRM function significantly attenuated cellular proliferation in this cell type. Collectively, these data establish a firm requirement of BAF57 for AR and co-activator function. Moreover, these data implicate BRM and BAF57 as putative therapeutic targets in prostate cancer therapy.

MATERIALS AND METHODS

Plasmids—pSG5AR (wild type androgen receptor), pSG5ARA70, pSG5ARA55, and pcDNA3-FLAGARA70N2 (22, 29, 73) expression plasmids were a gift from Dr. Chawnshang Chang (University of Rochester, Rochester, NY). pARR2-Luc was generated as previously described (43). pCR3.1 h-SRC1A was obtained from Dr. Bert O'Malley (Baylor College of Medicine, Houston, TX) (58). The pCMV-TIF2 mammalian expression construct was a gift from Dr. Alvaro Puga (University of Cincinnati, Cincinnati, OH). pFLAG-Ubc9 was received from Dr. Jorma Palvimo (University of Helsinki, Helsinki, Finland) (51). tk-Renilla luciferase was purchased from Promega and pcDNA3.1 from Invitrogen. pEGFP H2B-GFP was a gift from Dr. G. Wahl (Salk Institute, La Jolla, CA). pGEX-KG GST is from Dr. Robert Brackenbury (University of Cincinnati, Cincinnati, OH) (25). pGEX 3X GST-BAF57 was provided by Dr. Gail Mandel (SUNY at Stony Brook, Stony Brook, NY) and has been previously described (2). pCG-hBRM and pCG-dnBRM were provided by Dr. M. Yaniv (Institut Pasteur, Paris, France) (45). Dominant negative androgen receptor expression plasmid (pSG5AR Δ 46-408) was received from Dr. Ollie Janne (University of Helsinki, Helsinki, Finland) (49). pCMV-p16ink4a was previously described (40). pBabe-WTBAF57-FLAG and pBabe-BAF57 Δ N-FLAG were constructed by PCR amplification of each gene from the pBJ5 vector using primers that added the BamH1 (5') and EcoR1/FLAG (3'). The PCR product was digested and inserted into the pBabe vector (also encodes the puromycin resistance marker). The pHTP-BRMi construct employs a 64-mer double-stranded oligonucleotide to generate a shRNA targeting nucleotides 3388 to 3408 of the BRM mRNA. The fragment (or a scrambled control of the siRNA oligonucleotide sequence) was cloned into the pHTP-siRNA expression vector (a kind gift of Dr. William Reed, UNC-CH) using BglII and XhoI overhangs. The vector includes a puromycin resistance marker and places the expression oligo under the control of the human H1 promoter. pGreenLantern (Gibco) encodes the green fluorescent protein (GFP).

Immunohistochemistry—Prostates were resected from nine-week-old FVB/N mice. Individual lobes (ventral, dorsal-lateral, and anterior) were isolated, fixed by immersion in 10% neutral-buffered formalin and subsequently embedded in paraffin. Blocks were sectioned (5 μ m), mounted on slides, deparaffinized in xylene, and rehydrated in ethanol solutions. After antigen retrieval by boiling for 20 minutes in Antigen Unmasking Solution (Vector Laboratories), the sections were incubated for 15 minutes in Peroxidase Blocking Reagent (Dako Cytomation) to quench endogenous peroxidase. The sections were then blocked for 20 minutes in diluted normal goat serum (Rabbit IgG Vectastain ABC Kit; Vector Laboratories) then incubated with anti-BAF57c rabbit polyclonal antibody (1:1000 dilution in PBS solution) overnight at 4°C. After incubation for 30 minutes with anti-rabbit IgG biotinylated secondary antibody solution (Rabbit IgG Vectastain ABC Kit; Vector Laboratories), sections were incubated in ABC reagent for another 30 minutes. Antibodies were visualized with DAB Chromogen (DakoCytomation) and nuclei were counterstained with hematoxylin.

Tissue Culture and Treatment—BT459 cells were obtained from the ATCC and cultured in RPMI supplemented with 10% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 2mM L-glutamine, and 100 units/mL penicillin/streptomycin (Mediatech, Herndon, VA). For reporter analyses, BT549 cells were cultured in phenol red-free RPMI medium and 10% charcoal dextran treated FBS (CDT, Atlanta Biologicals, Norcross, GA). LNCaP cells received from the ATCC were cultured in IMEM supplemented with 5% heat inactivated FBS, 2mM L-glutamine, and 100 units/mL penicillin/streptomycin. LNCaP reporter assays were performed in phenol red-free IMEM supplemented with 5% CDT. MCF7, CV1, and SW13 cells were obtained from the ATCC and maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine, and 100 units/mL penicillin/streptomycin. Reporter analysis in these cell lines was carried out in phenol red-free medium containing 10% CDT.

LB1-luc cells were generated from the LNCaP cell line by co-transfection of the pARR2-Luc reporter construct (43) and the pBabe-PURO vector (which confers puromycin resistance). After selection in puromycin, individual colonies were isolated and screened for retention of ARR2 reporter activity. The resulting LB1-Luc cell line was tested by seeding 1×10^5 cells into a 12 well dish with IMEM medium containing 5% CDT. Cells were cultured in this condition of steroid deprivation for three days, and were then stimulated with the indicated concentrations of dihydrotestosterone (DHT, obtained from Sigma). Cells were harvested and measured for luciferase activity corrected against total protein (as determined via the BIORAD-DC assay, BioRAD).

Transfection and Reporter Analysis—MCF7, CV1, and SW13 cells were seeded in DMEM containing 10% CDT. Cells were transfected the following day using the BES/calcium phosphate protocol as previously described (8). Cells were transfected with 0.5 μ g pARR2-Luc, 0.5 μ g pSG5AR, 0.25 μ g pTK-renilla luciferase, and 2.25 μ g pBabe-WTBAF57-FLAG, 1 μ g pCG-hBRM, or parental vector (to a total of 4.5 μ g) as indicated. Post transfection, cells were treated with 0.1% ethanol vehicle (EtOH) or 0.1 nM dihydrotestosterone (DHT) for 24 hours. 0.1nM DHT was utilized as this is the optimal mitogenic dose for prostate cancer cells (0.1-1.0 nM DHT) (37). Cells were then harvested, lysed, and assayed for luciferase activity using the Dual Luciferase Assay System (Promega). For BT549 reporter analyses, cells were seeded in 6-well dishes with RPMI containing 10% CDT. Cells were transfected the following day using the FuGENE6 (Roche Molecular Biochemicals) method as recommended by the manufacturer. Transfections consisted of 0.5 μ g pARR2-Luc reporter, 0.5 μ g pSG5AR, and 2.25 μ g of pBabe-BAF57-FLAG (unless otherwise indicated) and/or 1 μ g of co-activator (pCR3.1-SRC1, pCMV-TIF2, pFLAG-Ubc9, pSG5ARA70, or pSG5ARA55) or 1 μ g of pCG-dnBRM or pSG5ARA70N2 as indicated. For each transfection, 0.25 μ g of pTK-renilla luciferase was used to normalize for transfection efficiency. Total plasmid DNA was brought to 4.5 μ g with the parental (empty)

vector. Following transfection, fresh media was added and cells were treated with either 0.1% EtOH or 0.1nM DHT for 24 hours. After stimulation, cells were harvested, lysed, and reporter analyses were carried out using the Dual Luciferase Assay Reporter System (Promega). LNCaP reporter analysis was carried out in 6-well dishes with IMEM containing 5% CDT. Cells were transfected using the Lipofectin (Invitrogen) transfection reagent as recommended by the manufacturer. LNCaP cells were transfected with 1 μ g pARR2-Luciferase reporter and 2.25 μ g pBabe-BAF57 Δ N-FLAG or parental vector (to 3.5 μ g total DNA). 0.25 μ g pTK-renilla luciferase was used to normalize for transfection efficiency. Following transfection, cells were stimulated with 1nM DHT. After 48 hours, cells were harvested and the Dual Luciferase Assay Reporter System was used to measure relative luciferase activity. Reporter assays were performed from at least in six independent transfections for each condition. Averages and standard deviations are shown. Statistical analysis was performed using the student t-test with a p-value of less than 0.05 indicating statistical significance. Transfection method was chosen based on the optimal method for each cell line (determined empirically using pTK-Renilla luc). Where indicated, parallel transfections were performed in which the internal control plasmid was replaced by pGreenLantern, and cells were harvested to monitor relative AR expression levels via immunoblot.

Immunoblotting—For protein analyses, cells were harvested by trypsinization (for mouse prostate, individual lobes were microdissected and ground), and lysis was carried out in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris [pH 8.0]) supplemented with protease inhibitors and phenylmethylsulfonyl fluoride. After brief sonication and clarification, equal protein was subjected to SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The membrane was immunoblotted with the indicated antibodies by standard techniques and visualized by chemiluminescence (Western Lightning,

PerkinElmer Life Sciences). The antibodies used were generated against BAF57c (gift from Dr. Weidong Wang), AR (N-20, Santa Cruz), GFP (B2, Santa Cruz) and CDK4 (H-22, Santa Cruz).

Immunoblotting to verify BRM knockdown via siRNA was carried out by transfection with 7 μ g of either pHTP-vector or pHTP-BRMi, along with co-transfection of 1 μ g pEGFP H2B-GFP into LNCaP cells. Post transfection, cells were selected using 0.625 μ g/mL puromycin (Sigma). Cells were simultaneously harvested after greater than 90% of the population was H2B-GFP positive (approximately 3 days). Pellets were rinsed in cold phosphate buffered saline (PBS), resuspended in buffer A (10mM Hepes [pH 7.9], 10mM KCl, 1.5mM MgCl₂, 0.5mM EDTA, 0.5mM DTT, 0.5mM PMSF, 0.5mg/mL leupeptin, and 1mg/mL aprotinin), and incubated on ice for 20 min. 6.5 μ l of 10% NP-40 was then added, the pellets vortexed, and centrifuged for 5 min. at 4°C. The supernatant was removed and the pellet resuspended in 50 μ l of cold Buffer C (20mM Hepes [pH 7.9], 400mM NaCl, 15mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, 0.5mg/ml leupeptin, and 1mg/ml aprotinin). Equal protein was subjected to 7.5% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Immunoblotting was performed with the indicated antibodies using standard techniques. Antibodies used were generated against BRM (courtesy of Dr. Moshe Yaniv), and actin (Sigma).

GST Pulldown—AR protein was produced and radiolabeled with [³⁵S]-methionine using the TnT-coupled Reticulocyte Lysate system (Promega) for *in vitro* transcription and translation according to the manufacturer's specifications and the NEG-772 Easytag Express Protein Labeling Mix (PerkinElmer Life Sciences). GST or GST-BAF57 was transformed into BL21 bacteria and induced using 0.1mM isopropyl- β -D-thiogalactopyranoside for 3 to 5 hours. After centrifugation, bacteria were washed one time in STE (150mM NaCl, 25mM Tris [pH 8.0], and 1mM EDTA) and resuspended in 3mL of STE. Pellets were lysed upon addition of 0.1 mg/mL lysozyme and 1.5% Sarkosyl, sonicated and clarified by centrifugation. Lysate was then incubated with 300 μ l of glutathione agarose beads at 4° overnight. Beads were washed 4 times

in PBS, then incubated with the *in vitro* translated protein for 3 hours at 4°C. Reactions were performed in 500µl NETN (20 mM Tris-HCl pH 8.0, 1mM EDTA, 100 mM NaCl, and 0.5% NP-40) supplemented with phenylmethylsulfonyl fluoride and protease inhibitors, and in the presence of either 0.1% EtOH or 10nM DHT, as indicated. “Input” (3% of total) and “Bound” fractions were separated by 10% SDS-PAGE, stained with Coomassie, and subjected to fluorography using Fluoro-Hance (Research Products International Corp., Mount Prospect, IL). Labeled protein was detected by autoradiography and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Co-immunoprecipitation—Immunoprecipitations were performed as previously described (28). LNCaP cells were harvested, washed in PBS, and lysed in IP buffer [20mM HEPES (pH 7.9), 250mM NaCl, 10% v/v glycerol, 0.1% v/v Tween20, 0.2mM EDTA, 2mM DTT] supplemented with protease inhibitors and 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 100ng of leupeptin and aprotinin per mL. After sonication and clarification, protein was quantified using the BIO-RAD DC protein assay kit. 1mg of protein was used for each immunoprecipitation reaction. Lysate was precleared with 1% pre-immune rabbit serum and 20µL protein-A Sepharose (Amersham) in 0.5mL of IP buffer for 1 hour at 4°C. Anti-AR, anti-BAF57, and pre-immune serum (negative control) were incubated with the lysate overnight at 4°C. 20µL protein-A Sepharose was added for a 1 hour incubation. Beads were subjected to 5 washes in IP buffer, resuspended in 2X SDS-PAGE buffer, and boiled to denature proteins. Samples were then subjected to SDS-PAGE and immunoblot analysis with antibody generated against AR (AR-N20, Santa Cruz).

Chromatin Immunoprecipitation—LNCaP or LB1-Luc cells were seeded into 15cm dishes in phenol red-free IMEM supplemented with 5% CDT. After four days of incubation, cells were stimulated with either 0.1% EtOH or 10nM DHT for 60 minutes. A higher than physiological dose was utilized for ChIP analysis (consistent with the literature) in order to enhance AR on

target promoters (59). Cells were then treated with 1% formaldehyde diluted in phosphate buffered saline to allow for protein cross-linking to DNA, followed by quenching with 0.125M glycine. Cells were then washed with ice-cold PBS, trypsinized, and pelleted. Pellets were washed in PBS containing 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 100ng of leupeptin and aprotinin per mL. Cells were lysed in cell lysis buffer (5mM PIPES [pH 8.0], 85mM KCl, 0.5% NP-40, 0.5mM PMSF, 100ng of leupeptin and aprotinin per mL) for 10 minutes. Nuclei were lysed in 400 μ L of nuclei lysis buffer (1% sodium dodecyl sulfate [SDS], 50mM Tris-Cl [pH 8.1], 10mM EDTA, 0.5mM PMSF, 100ng of leupeptin and aprotinin per mL) for 10 min., and sonication was performed to shear DNA. Immunoprecipitations were carried out using 80 μ L of lysate and antibodies to AR (N-20, Santa Cruz), acetylated histone H4 (06-866, Upstate Biotechnology), BAF57c (specific for c-terminus), BAF57h (specific for HMG domain) (gift from Dr. Weidong Wang) (71), and a control IgG antibody (pre-immune sera). Chromatin and antibody were incubated in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris [pH 8.0]) for 1.5 hours at 4°C, at which time protein-A Sepharose (Amersham) was added for an additional incubation. Beads were washed three times with super RIPA (RIPA + 300mM NaCl), once with RIPA, and once with TE buffer (10mM Tris [pH 8.0], 1mM EDTA) with 10 minute rotations between each wash at room temperature. 300 μ L ChIP extraction buffer (1% SDS, 0.1M NaHCO₃) was then added, along with 20 μ L 5M NaCl, and RNase A. Cross-linking was reversed by incubation at 65°C (4 hours up to overnight). DNA was purified using the QIAquick PCR Purification Kit (Qiagen). PCR amplification was performed on recovered DNA products as well as input chromatin (10% of input for Fig. 6A, 6C; 1% of input in Fig. 6B) in the presence of alpha³²P-dCTP, which allows accurate quantification of the PCR products over a broad range of DNA concentrations (60). To monitor recruitment to the PSA enhancer, PCR was performed using G/H primers, as previously described (59). These primers are specific for a region containing an androgen responsive element (AREIII) contained within

the enhancer region of the prostate specific antigen gene. For PCR parameters were 94°C for 5 min.; 28 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and 72°C for 10 minutes. To monitor recruitment to the probasin promoter, the following primers were utilized: forward- 5'-CATCTACCATTCCAGTTAAG and reverse- 5'-CCAGCGGTTCATCC. PCR parameters were 94°C for 5 min, followed by cycles of: 94°C for 45 s, 49°C for 45s, and 72°C for 45 s. PCR products were separated on a 6% polyacrylamide gel and visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Reverse Transcriptase PCR—LNCaP cells were seeded in 6cm dishes and transfected with the Lipofectin transfection reagent with 1 μ g pEGFP H2B-GFP to monitor for transfection efficiency, 1 μ g pBabe-PURO for rapid puromycin selection, and 4 μ g of one of the following expression plasmids: pCMV-NEO BAM (vector control), pBabe-BAF57 Δ N-FLAG, or dominant negative androgen receptor (pSG5AR Δ 46-408, positive control). After transfection, cells were allowed to recover for approximately 24 hours. Transfected cells were selected using 0.625 μ g/mL puromycin (Sigma). Selection was carried out until greater than 90% of cells were H2B-GFP positive (approximately 3 days). RNA was isolated using the TRIzol Reagent (Gibco) as recommended by the manufacturer. To quantify expression, RT-PCR was performed with amplification of the cDNA performed in the presence of ³²P-dCTP to quantify expression over a large linear range, as previously described (60). Primers were used for prostate specific antigen (PSA) (primer pair: 5'-CTTGTAGCCTCTCGTGGCAG-3'; 5'-GACCTTCATAGCATCCGTGAG-3') and GAPDH (loading control) (primer pair: 5'-CCACCCCATGGCAAATTCCATGGCA-3'; 5'-TCTAGACGGCAGGTCAGGTCCACC-3'). PCR conditions were as follows: 94°C for 2 min.; 23 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and 72°C for 10 min. PCR reactions were separated by polyacrylamide gel electrophoresis and signal was quantified on a PhosphorImager. For comparison, maximal AR activity (PSA/GAPDH) in the presence of vector was set to 100. Representative data from two independent experiments is shown.

Bromodeoxyuridine incorporation—LNCaP cells were seeded onto poly-L-lysine coated coverslips, followed by transfection using Lipofectin, as described above. Cells were transfected with 0.5 μ g H2B-GFP along with 4.5 μ g of one of the following expression plasmids: pBabe-PURO, pBabe-BAF57 Δ N-FLAG, dominant negative androgen receptor (pSG5AR Δ 46-408), pHTP vector, pHTP-scrambled, pHTP-BRMi, or pCMV-p16ink4a. Post transfection (3 days for the pBabe-PURO/BAF57 Δ N/dnAR experiment or 4 days for the pHTP-vector/pHTP-BRMi/pCMV-p16ink4a experiment) bromodeoxyuridine (BrdU, Amersham Biosciences), labeling reagent was added for a 16 hour incubation. Detection of BrdU by indirect immunofluorescence was performed as previously described (41). A minimum of 150 H2B-GFP transfected cells per sample were counted for BrdU incorporation. Results are plotted as percent inhibition over vector control and the average of at least three replicates from two independent experiments is shown.

RESULTS

BAF57 is expressed in the prostatic epithelia. We and others have previously shown that SWI/SNF function is required for AR activity, and that AR is preferentially assisted by the BRM-containing SWI/SNF complex (30, 32, 43). Correspondingly, BRM (but not BRG1) has been detected in normal human prostatic epithelia (53). However, the mechanism by which SWI/SNF is recruited to AR targets remained unsolved. Similar to the inability of the core ATPase, BRG1, to bind to the estrogen and glucocorticoid receptors (14, 28), we failed to observe *in vitro* interaction of BRM with recombinant AR (data not shown), thus indicating that AR does not bind directly to the core ATPase. It has been previously demonstrated that selected SWI/SNF subunits may modulate association with sequence-specific binding to transcription factors. For example, the glucocorticoid receptor (GR) has been shown to interact directly with BAF250, BAF60a, and BAF57 (28, 47). The BAF57 subunit was also shown to interact with both the estrogen receptor (ER) and p160 co-activators, and was critical for p160 function in these assays (3). Here, we demonstrate that BAF57 is highly expressed in prostatic epithelia. As can be seen in Fig. 1A, BAF57 was detected in all lobes of the murine prostate (lanes 3-5). To ascertain the cellular origin of BAF57 expression in the prostate, immunohistochemistry was performed (Fig. 1B). As is observed, BAF57 was predominantly expressed in the luminal epithelial layer and was enriched in the nuclei (right two panels, top 20X, bottom 40X), compared to the no primary antibody control (left two panels, top 20X, bottom 40X). These observations indicate that BAF57 is highly expressed in the AR positive, AR dependent prostatic epithelia. Given the requirement of SWI/SNF for AR function, a potential relationship between BAF57 and AR was investigated.

BAF57 binds directly to the AR and is associated with AR in prostate cancer cells. To examine the putative role of BAF57 in AR function, GST pull-down assays were employed (Fig. 2A). Recombinant AR bound significantly to GST-BAF57 (as compared to GST control), as in

the absence of ligand approximately 60% of input was captured, compared to only 7% with GST (compare lanes 2, 3). Inclusion of ligand did not enhance AR binding (compare lanes 3, 5). Thus, these data indicate that BAF57 can directly bind AR, independent of ligand. To verify these findings, co-immunoprecipitations were performed using AR-positive prostate cancer cells (LNCaP). Cell lysate was subjected to immunoprecipitation analysis using antibodies to AR, BAF57, and pre-immune serum, as indicated. Immunoblotting with antibody to AR revealed an endogenous interaction between BAF57 and the AR (Fig. 2B). Combined, these data demonstrate that AR associates with BAF57 in prostate cancer cells.

BAF57 is required for receptor activity. To assess the functional role of BAF57 on AR activity, a cell line deficient in BAF57 (BT549) was utilized. Although null for BAF57 (Fig. 3A), these cells do express other core SWI/SNF proteins (including both BRM and BRG1) (3, 13). By contrast, MCF7, LNCaP, and CV1 cells are competent for both BAF57 expression (Fig. 3A) and SWI/SNF function (43). To compare relative AR activity in these lines, reporter assays were employed using a reporter for a well-described AR target gene, probasin (pARR2-Luc) (76). Transfections and reporter analyses were performed as indicated in the Materials and Methods section. As can be seen in Fig. 3B, MCF7 and CV1 cells demonstrated robust AR activity (63-fold and 65-fold induction, respectively). LNCaP cells also exhibited strong activation of AR target genes (Fig. 4A) (11, 76). By contrast, in the BAF57 deficient BT549 cell line, AR activation was significantly impaired (only 4 fold increase over EtOH control) (Fig. 3B). This deficiency was not attributed to a lack of ectopic AR expression, as determined by immunoblot (Fig. 3C, compare lanes 1, 2) (GFP expression denotes transfection). To establish the role of BAF57 in this aberrant response, reporter analyses were repeated in the presence of restored BAF57. Addition of BAF57 to BT549 cells restored ligand dependent AR activation (Fig. 3D), indicating a functional role for BAF57 in regulating AR activity. The difference in pARR2-Luc activity was not attributed to altered AR expression levels, as parallel experiments using a GFP

expression plasmid (pGreenLantern) as an internal control demonstrated equal AR protein levels in the presence and absence of BAF57 (Fig. 3E, compare lanes 1, 2).

Abrogation of BAF57 results in reduction of endogenous AR activity. To further elucidate the function of BAF57 on AR target gene transcription, BAF57 activity was abrogated in the AR positive prostatic adenocarcinoma cell line, LNCaP. These cells are dependent on AR for proliferation and express BAF57 (Fig. 3A). A well-characterized dominant negative allele of BAF57, BAF57 Δ N, was utilized to suppress endogenous function. BAF57 Δ N lacks the proline-rich region and the HMG domain (N-terminus) and has been shown to block BAF57 activity and downregulate endogenous BAF57 expression (via immunoblot analysis) in the thymocytes of BAF57 Δ N transgenic mice (9). Importantly, this allele has been shown to retain association with SWI/SNF. For these experiments, LNCaP cells were transfected in the absence of ligand with pARR2-Luc and either pBabe-BAF57 Δ N or vector control to monitor impact on endogenous AR activity (Fig. 4A). As expected, ligand induced robust activation of the pARR2-Luc target gene by endogenous AR in the presence of empty vector (28-fold over basal activity). By contrast, there was a significant reduction in endogenous AR activation of the reporter upon transfection of pBabe-BAF57 Δ N (approximate 2-fold reduction in activity), thereby demonstrating the importance of BAF57 for endogenous AR function. Furthermore, endogenous AR levels remained constant upon transfection of vector control and pBabe-BAF57 Δ N, relative to control (CDK4) (Figure 4B). Transient introduction of pBabe-BAF57 Δ N into LNCaP cells demonstrated no effect on BAF57 expression levels (data not shown), thus indicating that in this system, BAF57- Δ N functions through its defined dominant negative capabilities. Ectopic expression of wild-type BAF57 has been previously demonstrated to also act in a dominant negative fashion in cell lines containing endogenous BAF57, presumably by titrating endogenous complexes (3). Combined, our observations indicate that abrogation of BAF57 activity reduces AR function.

To verify this hypothesis, the effect of BAF57 Δ N on an endogenous AR target gene (prostate specific antigen, PSA) was examined. For these experiments, LNCaP cells were transfected with pBabe-puro (encoding a puromycin resistance marker), pEGFP H2B-GFP (to monitor transfection/selection), and either vector control, pBabe-BAF57 Δ N, or pSG5-AR Δ 46-408 (dominant negative AR) as a positive control. After rapid selection with puromycin, RNA was isolated, and after reverse-transcription, PCR amplification was performed in the presence of ³²P-dCTP to examine PSA mRNA expression (Figure 4C). As expected, dnAR significantly reduced PSA mRNA expression, relative to vector control (upper panel, compare lanes 1, 3). Strikingly, BAF57- Δ N similarly attenuated PSA expression (upper panel, compare lanes 1, 2). Quantification of these data indicate that BAF57- Δ N inhibits PSA expression similar to dominant-negative AR (lower panel). Together, these data confirm that disruption of BAF57 function abrogates AR activity on both exogenous and endogenous AR targets.

SWI/SNF ATPase activity is required for BAF57 function. Although these data indicate a requirement of BAF57 for AR transactivation potential, the necessity of SWI/SNF ATPase activity for BAF57 function has not been demonstrated. This point is particularly salient, as BAF57 can bind DNA independent of the SWI/SNF complex (71). Therefore, we initially tested the ability of BAF57 to promote AR function in coordination with the preferred core ATPase, BRM, in BT549 cells. Although null for BAF57, BT549 cells have been shown previously to express the BRM ATPase (13). Ectopic expression of dominant negative BRM (pCG-dnBRM) was utilized to examine the impact of the core ATPase in the absence of BAF57. This allele of BRM has been previously described (45) and encodes an ATPase defective mutant protein which can still assemble into SWI/SNF, but fails to remodel chromatin. Consistent with our previous observations (Fig. 3D), ligand induced a similar fold induction (8-fold) over basal activity (EtOH control) (Fig. 5A). Moreover, dnBRM failed to promote receptor activity, and slightly inhibited residual AR activity. This may be indicative of the ability of SWI/SNF to regulate

AR activity even in the absence of BAF57 (albeit inefficient). As expected, BAF57 restored AR activation (Fig. 5A). However, BAF57-mediated AR activation was significantly reduced upon expression of dnBRM, thus indicating that BAF57 requires SWI/SNF ATPase function to efficiently promote AR activity. Parallel experiments using GFP expression as an internal control demonstrated that expression of dnBRM had no impact on AR protein levels (Fig. 5B). To verify the requirement of BAF57 for SWI/SNF function, SW13 cells were utilized. These cells are null for both BRG1 and BRM, and thus lack SWI/SNF activity. However, these cells do express BAF57 (13). Consistent with our published findings (43), SW13 cells failed to support significant AR activity in reporter assays (Fig. 5C), however expression of pCG-BRM restored AR function. Moreover, BAF57 failed to promote AR activity in this model system, thus demonstrating that BAF57 action is dependent on a functional SWI/SNF complex.

BAF57 is recruited to AR target regulatory regions in the presence of ligand. Having demonstrated the requirement of BAF57 for AR function, we examined the recruitment of BAF57 to paradigm AR targets in the prostate using chromatin immunoprecipitation (ChIP). Isolated chromatin from LNCaP cells was immunoprecipitated with antibodies directed against either AR, BAF57 [BAF57h (Fig. 6B), directed against the HMG domain, or BAF57c (Fig. 6C), directed against the C-terminus], acetylated histone H4 (AcH4), or a pre-immune serum. Recruitment of protein to the prostate specific antigen (PSA) regulatory region was determined using primers specific to the PSA enhancer encompassing a well-defined androgen responsive element (ARE) (11, 59). A similar result was observed at an ARE contained within the proximal PSA promoter (data not shown). PCR amplification was performed in the presence of ³²P-dCTP and quantified using autoradiography (Fig. 6A and 6B, right panels), as previously described (60). As shown (Fig. 6A), minimal AR or acetylated histone H4 could be detected at the PSA enhancer in the presence of EtOH vehicle. However, upon ligand stimulation, AR was recruited to the PSA enhancer, and histone acetylation was apparent (compare lanes 1, 2). These data are consistent with previously published reports (59). As shown in Figure 6B, BAF57 was

detected at the PSA enhancer, and was significantly enriched after ligand stimulation (compare lanes 1, 2). To further confirm the recruitment of BAF57 to AR targets, ChIP analyses were repeated using an LNCaP derived cell line, LB1-Luc. LB1-Luc cells contain a stably integrated pARR2-Luciferase promoter, as described in the Materials and Methods section. As can be seen in Figure 6C, these cells exhibit robust AR activity in a dose dependent manner upon addition of ligand (left panel). ChIP analysis using isolated chromatin from the LB1-Luc cells reveals the recruitment of AR and BAF57 to the pARR2-Luc promoter upon stimulation with DHT (right two panels). Taken together, these data confirm the presence of BAF57 on AR regulatory regions in the presence of ligand.

BAF57 works cooperatively with p160 HAT co-activators to activate transcription of AR target genes. A role for BAF57 has been previously established in p160 histone acetyltransferase (HAT) co-activator mediated function, using ER as a model (3). Interestingly, p160 co-activators have been shown to target histones H3 and H4 for acetylation (38, 42, 64). Since BAF57 recruitment was observed when histones were acetylated (Fig. 6), we analyzed the impact of BAF57 in coordination with p160 co-activators (Figure 7) to enhance AR activity. For these experiments, reporter analyses were performed in BT549 cells as in Figure 3. As shown, SRC1 expression failed to generate significant AR activity in the presence of ligand compared to vector control. As expected, BAF57 restored significant AR activity, consistent with our previous results (Fig. 3D). However, the combination of SRC1 and BAF57 led to markedly enhanced AR activity (10.3 fold over SRC1 alone and 2.1 fold over BAF57 alone) (Fig. 7A). Interestingly, unlike SRC1 alone, TIF2 by itself appeared to significantly enhance AR activity similar to BAF57 alone (Fig. 7B). However, a similar profile to SRC1 was observed upon co-transfection of TIF2 and BAF57 (3.2 fold over TIF2 alone and 2.5 fold over BAF57 alone). These data indicate that BAF57 may cooperate with p160 co-activators to enhance AR activity.

BAF57 facilitates non-p160 AR co-activator function. Upon establishing a role for BAF57 in p160 co-activator AR function, BAF57 influence on non-p160 co-activator function was

examined. BT549 cells were utilized, and transfected with the indicated effector plasmids as above. Interestingly, ARA70 supported a dose dependent response to BAF57 (Figure 8A). A significant induction of AR activity was stimulated by addition of both ARA70 and BAF57 (versus BAF57 alone) at higher concentrations of BAF57, as indicated. A more than additive effect was observed at the highest doses [8.4 fold over ARA70 alone (2 fold over EtOH, data not shown) and 3.6 fold over BAF57 alone]. By contrast, a mutant of ARA70 which retains the ability to interact with AR through the FXXLF AR interacting motif but lacks the ability to co-activate AR activity (ARA70N2) failed to cooperate with BAF57 or induce AR activity (29) (Figure 8B). These observations indicate a functional co-activator is necessary for enhanced AR activation by BAF57. As shown in Figure 8C, BAF57 also facilitated ARA55 activity, stimulating AR activity 3.5-fold over ARA55 alone and 4.2-fold over BAF57 alone (Fig. 8C). Lastly, the impact of BAF57 on the E2 SUMO-conjugating enzyme Ubc9 was assessed. Ubc9 activates AR in a manner independent of its ability to conjugate SUMO-1 to AR, through poorly defined mechanisms (51). Similar to the selected SRC1, Ubc9 alone failed to significantly activate AR target gene transcription, but an enhanced effect was observed in combination with BAF57 (5 fold over Ubc9 alone and 1.7 fold over BAF57 alone), indicating BAF57 is able to facilitate Ubc9 co-activator function (Fig. 8D). Together, these data indicate that the Ubc9 requirement for BAF57 is similar to that of the p160 co-activators. By contrast, while ARA70 and ARA55 do not require BAF57, they synergize with BAF57 for AR activation.

Abrogation of either BRM or BAF57 attenuates AR dependent LNCaP cell proliferation.

Our studies demonstrate a specific requirement of BAF57 for both ligand induced and co-activator enhanced AR activation. Since AR activity is essential for the proliferation of prostate cancer cells, we examined the necessity of both BRM and BAF57 in this process. Initially, BAF57 function was depleted via transfection of the dominant negative expression plasmid (pBabe-BAF57 Δ N) into LNCaP cells. This approach was utilized, as attempts to target BAF57 using siRNA technology have not yet proven successful (data not shown). This experiment was

performed by transfection of LNCaP cells with pEGFP-H2B-GFP (to mark transfected cells) and either parental vector, pBabe-BAF57 Δ N, or pSG5AR Δ 46-408 (dnAR). As expected, dnAR attenuated LNCaP cell proliferation (34% inhibition) (Fig. 9A, right panel). Representative data from two independent experiments is shown in Fig. 9A (left panel), and demonstrate that cells transfected with pBabe-BAF57 Δ N were dramatically inhibited for BrdU incorporation. Percent inhibition of BrdU incorporation over vector control was measured, as can be seen in Fig. 9A (right panel). Interestingly, abrogation of BAF57 slows proliferation of LNCaP cells (35% inhibition) over vector control in a manner similar to the positive control, dnAR.

To examine the role of the ATPase, BRM expression was targeted using siRNA technology. Efficacy of the construct was demonstrated by immunoblotting as seen in Fig. 9B, wherein LNCaP cells were transfected with pHTP parental vector or pHTP-BRMi (both of which confer puromycin resistance), as indicated, and immunoblotted to detect endogenous BRM. As shown, transfection of pHTP-BRMi resulted in significant knockdown of endogenous BRM in LNCaP cells compared to vector (compare lanes 2, 3). SW13 cells were included as a negative control and actin was detected to demonstrate equal loading. The effect of BRM ablation on LNCaP cellular proliferation was subsequently assessed by BrdU incorporation (Fig. 9C). Cells were transfected as indicated, and pEGFP-H2B-GFP positive (transfected) cells were screened for BrdU incorporation and impact on proliferation plotted as percent inhibition of BrdU incorporation over vector control (Fig. 9C). As expected, expression of p16ink4a significantly abrogated LNCaP cell growth (4.1 fold over vector and 4.7 fold over scrambled). Proliferation of LNCaP cells transfected with expression plasmid for the BRM siRNA were significantly reduced compared to vector control (3.9 fold reduction) or scrambled BRM siRNA control (4.4 fold reduction). Thus, ablation of BRM severely retards LNCaP cellular proliferation. Together, these data clearly establish a requirement of both BAF57 and the BRM ATPase for proliferation of AR-dependent prostate cancer cells.

DISCUSSION

The SWI/SNF chromatin remodeling complex is involved in activating gene transcription of nuclear receptor targets. We have previously shown that AR requires SWI/SNF for activity, although the mechanisms underlying this requirement remained obscure. Here, we identify the BAF57 subunit as a major regulator of SWI/SNF effects on the AR in the prostate. We show that BAF57 is expressed in the AR-positive luminal epithelia of the prostate (Fig. 1), which are dependent on serum androgen for proliferation and survival. Subsequently, a ligand independent *in vitro* interaction was observed between BAF57 and AR, and AR was found to be in association with BAF57 *in vivo* (Fig. 2). Combined, these data indicated that BAF57 may be the mechanism by which SWI/SNF interfaces with the AR. Consistent with this hypothesis, cells deficient in BAF57 exhibited significant defects in AR activity, and rescue of BAF57 in such cells effectively restored AR activity (Fig. 3). These findings were confirmed in AR-dependent prostate cancer cells, wherein expression of the endogenous AR target, PSA, was attenuated to a level similar to that observed with dominant negative AR (Fig. 4). Collectively, these observations define a requirement of BAF57 for AR transactivation potential. This function of BAF57 was shown to be dependent on SWI/SNF function, as either loss or abrogation of BRM ATPase activity significantly compromised BAF57 action (Fig. 5). Although the *in vitro* interaction between AR and BAF57 occurred independent of ligand, we show that BAF57 is recruited to endogenous AR targets in response to ligand activation, thus supporting the hypothesis that BAF57 regulates ligand-dependent AR activation (Fig. 6). The impact of BAF57 was not limited to AR alone, as BAF57 also influenced AR co-activator function. Active BAF57 was essential for AR potentiation exerted by either SRC1 or Ubc9, and moreover BAF57 cooperated with this selected subset of co-activators for AR activation. By contrast, while BAF57 was not essential for TIF2, ARA70, or ARA55 AR co-activator function, BAF57 facilitated the ability of these co-activators to induce AR activation (Fig. 7, 8). Given the observed requirement

of BAF57 for AR and co-activator function, we examined the requirement of BAF57 and SWI/SNF for proliferation. Strikingly, ablation of either BAF57 or BRM function severely retarded proliferation in AR-dependent prostate cancer cells (Fig. 9). Thus, these data establish a critical role for BAF57-mediated SWI/SNF function in AR-dependent cells.

Although the SWI/SNF complexes are widely expressed, their function results in a multitude of diverse biological outcomes. For example, association of SWI/SNF with the GR is required for GR-mediated transcriptional activation (45, 74). By contrast, both core ATPase subunits (BRG1 and BRM) associate with the retinoblastoma tumor suppressor protein, RB, to regulate RB-dependent transcriptional repression of E2F target genes. This mechanism of repressive action is controversial in that it has been demonstrated to occur through direct interaction between the ATPase and RB (15, 54, 65, 66, 75), as well as through up-regulation of the cdk inhibitor p21cip1 (36). This function of RB is essential for its anti-proliferative function, and loss of SWI/SNF association renders all tested cells resistant to RB-mediated growth arrest (54, 66). Thus, SWI/SNF can confer either transcriptional activation or repression. Specificity for SWI/SNF action is purported to be governed by the biochemical diversity and plasticity of SWI/SNF subunit composition, wherein selected subunits likely regulate tissue or cell-type specific protein-protein interactions.

Targeted deletion of the core ATPases in mouse model systems first revealed non-redundancies in SWI/SNF action. Deletion of BRG1 is an embryonic lethal event (7), whereas BRM $-/-$ mice are viable and demonstrate increased body weight (55). Intriguingly, BRM null mice exhibited slightly reduced testicular weight, indicating a putative defect in androgen-dependent development (55). Specificity for the ATPase has also been shown biochemically, wherein BRG1 was shown to preferentially regulate selected zinc finger proteins, whereas BRM interacted preferentially with members of the Notch pathway (35). Variations in cell type expression may partially underlie specificity of ATPase action, as BRG1 is primarily expressed in proliferating or self-renewing cells, and BRM protein levels are enriched in differentiated cell

types. Differentiated prostatic epithelia and prostatic adenocarcinoma cells require AR for survival, and consistent with the role of BRM in such cell types, we have shown that the AR is preferentially activated by BRM-containing SWI/SNF complexes (43). Furthermore, in support of this observation, normal human prostate epithelia were recently shown to express the BRM ATPase; in contrast, BRG1 was not detected (53). Thus, the core ATPase lends some specificity to SWI/SNF modulation of the AR. However, no direct interaction between the AR and BRM was observed *in vitro* (data not shown), thus indicating that SWI/SNF action must be either indirect or through association with alternate SWI/SNF subunits.

The ability of SWI/SNF subunits to direct the complex to sequence-specific transcriptional complexes and thus regulate discrete biological processes has strong precedent. For example, loss of the SNF5 subunit is associated with human rhabdoid tumorigenesis, and SNF5 *-/-* mice develop lymphomas or rhabdoid tumors with complete penetrance (56). SNF5 tumor suppressor activity is thought to occur in part through the cell cycle inhibitor p16ink4a (5, 48). The BAF250 subunit, which contains intrinsic DNA binding capabilities through an AT-rich DNA binding domain, directs SWI/SNF activity to the GR (47). Thus, BAF250 may utilize both DNA and protein binding functions in combination to confer specificity of action. By contrast, BAF60a likely utilizes two distinct protein binding surfaces (the first for GR and BRG1, and the second for BAF155 and BAF170) to regulate GR specificity (28). BAF60a has also been shown to function as to discriminately activate a subset of Fos/Jun dimers, thus governing specificity of AP-1 action (33). Lastly, BAF57 harbors multiple activities which likely assist in the regulation of sequence-specific DNA binding factors (2, 3, 71).

BAF57 is composed of an N-terminal high mobility group (HMG) DNA binding domain and a kinesin-like coiled coil region. Given this structure and its ability to bind 4-way junction DNA, BAF57 has been suggested as a potential targeting subunit of the SWI/SNF complex that regulates selected target genes (71). This hypothesis has been tested using the Δ HMG allele of BAF57, which does not disrupt SWI/SNF complex formation or SWI/SNF recruitment, but rather

lacks DNA binding potential. Transgenic mice expressing this allele in T-cells demonstrate a specific de-repression of the CD4 locus (9). Here, we report that BAF57 interacts directly with the AR, and activates AR function through SWI/SNF. Although AR can bind BAF57 independent of ligand (similar to what was reported for GR (28)), BAF57 was recruited to AR targets in a ligand-dependent manner. As we have also observed that BAF57 resides predominantly in the nucleus (data not shown), interaction between AR and BAF57 is hypothesized to occur in this cellular compartment. Mechanisms regulating the AR-BAF57 interaction will be the subject of future studies. The ER has also been shown to interact with BAF57, but ER binding is enhanced by ligand, thus indicating a disparate mode of interaction. Supporting this view, ER was shown to retain transactivation capacity in BAF57 null cells (3), whereas AR activity was markedly reduced by BAF57 loss or ablation. The requirement of BAF57 for AR was apparent in both transient assays and upon analysis of PSA, an endogenous AR target gene. It is of interest to note that previous analyses of SWI/SNF effects on this AR target revealed that in transient assays with an ectopically expressed PSA regulatory region, presence of the PSA enhancer relieved the requirement of SWI/SNF for AR function (43), thus indicating that DNA composition or architecture likely influences the necessity for SWI/SNF. Although such studies are useful for dissecting the mode of SWI/SNF action on discrete templates, the data shown herein establish a solid requirement for BAF57 and SWI/SNF action on prostate specific AR targets in the context of chromatin.

Independent of nuclear receptor association, BAF57 has been shown to regulate co-activator function. Specifically, BAF57 has been shown to interact directly with the p160 co-activators, and is required for the ability of these co-activators to modulate ER function (3). Similarly, we show SRC1 maintains little AR co-activation function in the absence of BAF57, and cooperates with BAF57 to augment AR activity. In contrast to SRC1, TIF2 maintains activity in the absence of BAF57, however addition of BAF57 facilitates AR action similar to SRC1. Surprisingly, potentiation of AR by Ubc9 demonstrated a similar requirement, indicating that the

BAF57 requirement extends beyond the p160 class of co-activators. Although the mechanism by which Ubc9 modulates AR remains elusive, these data put forth the hypothesis that Ubc9 may activate p160 function. By contrast, ARA70 and ARA55 potentiated AR gene transcription independent of BAF57, and actually synergized with BAF57 for AR activation. Thus, this class of co-activators maintain function independent of SWI/SNF. Collectively, these data indicate that like the nuclear receptors, co-activators demonstrate discrete requirements for SWI/SNF function. Given the importance of selected co-activators in prostate cancer progression, knowledge of SWI/SNF requirement will be an important component of targeting co-activator function in cancer therapy.

Ablation of AR activity is the main line of treatment for prostate cancer therapy, and recurrent, late stage tumors are hallmarked by inappropriate restoration of AR activity (1, 34). We have shown that ablation of either BRM or BAF57 significantly retards proliferation in prostate cancer cells. These observations again underscore the plasticity of the SWI/SNF requirement, as loss of SWI/SNF ATPase activity or selected subunits is often associated with growth advantage, such as is observed upon SNF5 loss or in response to RB upon loss of SWI/SNF ATPase activity (5, 54, 56, 66, 69). Together, these data implicate a specific response for BAF57 in AR dependent growth and further identify the importance of the BAF subunits in SWI/SNF function.

In summary, these data reveal an essential role for BAF57 in regulating SWI/SNF modification of AR. Moreover, our studies demonstrate that BAF57 strongly influences the impact of co-activators on AR activation. These functions of BAF57 are critical, as ablation of BAF57 or BRM function blocked proliferation of androgen-dependent prostate cancer cells. Together, these studies implicate BAF57 as a major regulator of AR and prostate cancer proliferation, thus identifying BAF57 as a valid target for potential therapeutic intervention.

ACKNOWLEDGMENTS

We thank Dr. Weidong Wang for the generous gift of the BAF57 antibodies. We thank Drs. Erik Knudsen, Christin Petre, and Chris Mayhew, also Hasan Siddiqui, and Janet Hess-Wilson for critical reading of the manuscript. We thank Dr. Robert Matusik, Tom Case, and Dr. Janni Mirosevich for training in prostate tissue microdissection and immunohistochemistry. This work was supported by DAMD 02-1-0037 (to K.E.K.) and RO1CA099996 (to K.E.K.). Bernard Weissman, Gary Rosson, and Erin Henry are supported by grants CA91048 and CA102848. Kevin Link is supported by NIEHS Training Grant 2 T32 ES07250-16.

REFERENCES

1. **Balk, S. P.** 2002. Androgen receptor as a target in androgen-independent prostate cancer. *Urology* **60**:132-8; discussion 138-9.
2. **Battaglioli, E., M. E. Andres, D. W. Rose, J. G. Chenoweth, M. G. Rosenfeld, M. E. Anderson, and G. Mandel.** 2002. REST repression of neuronal genes requires components of the hSWI.SNF complex. *J Biol Chem* **277**:41038-45.
3. **Belandia, B., R. L. Orford, H. C. Hurst, and M. G. Parker.** 2002. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *Embo J* **21**:4094-103.
4. **Belandia, B., and M. G. Parker.** 2003. Nuclear receptors: a rendezvous for chromatin remodeling factors. *Cell* **114**:277-80.
5. **Betz, B. L., M. W. Strobeck, D. N. Reisman, E. S. Knudsen, and B. E. Weissman.** 2002. Re-expression of hSNF5/INI1/BAF47 in pediatric tumor cells leads to G1 arrest associated with induction of p16ink4a and activation of RB. *Oncogene* **21**:5193-203.
6. **Brinkmann, A. O., L. J. Blok, P. E. de Ruiter, P. Doesburg, K. Steketee, C. A. Berrevoets, and J. Trapman.** 1999. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* **69**:307-13.
7. **Bultman, S., T. Gebuhr, D. Yee, C. La Mantia, J. Nicholson, A. Gilliam, F. Randazzo, D. Metzger, P. Chambon, G. Crabtree, and T. Magnuson.** 2000. A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. *Mol Cell* **6**:1287-95.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7**:2745-52.
9. **Chi, T. H., M. Wan, K. Zhao, I. Taniuchi, L. Chen, D. R. Littman, and G. R. Crabtree.** 2002. Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature* **418**:195-9.
10. **Chiba, H., M. Muramatsu, A. Nomoto, and H. Kato.** 1994. Two human homologues of *Saccharomyces cerevisiae* SWI2/SNF2 and *Drosophila* brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucleic Acids Res* **22**:1815-20.
11. **Cleutjens, K. B., H. A. van der Korput, C. C. van Eekelen, H. C. van Rooij, P. W. Faber, and J. Trapman.** 1997. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol* **11**:148-61.
12. **Collingwood, T. N., F. D. Urnov, and A. P. Wolffe.** 1999. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol* **23**:255-75.
13. **Decristofaro, M. F., B. L. Betz, C. J. Rorie, D. N. Reisman, W. Wang, and B. E. Weissman.** 2001. Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. *J Cell Physiol* **186**:136-45.
14. **DiRenzo, J., Y. Shang, M. Phelan, S. Sif, M. Myers, R. Kingston, and M. Brown.** 2000. BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. *Mol Cell Biol* **20**:7541-9.
15. **Dunaief, J. L., B. E. Strober, S. Guha, P. A. Khavari, K. Alin, J. Luban, M. Begemann, G. R. Crabtree, and S. P. Goff.** 1994. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79**:119-30.

16. **Feldman, B. J., and D. Feldman.** 2001. The development of androgen-independent prostate cancer. *Nat Rev Cancer* **1**:34-45.
17. **Flaus, A., and T. Owen-Hughes.** 2001. Mechanisms for ATP-dependent chromatin remodelling. *Curr Opin Genet Dev* **11**:148-54.
18. **Fletcher, T. M., N. Xiao, G. Mautino, C. T. Baumann, R. Wolford, B. S. Warren, and G. L. Hager.** 2002. ATP-dependent mobilization of the glucocorticoid receptor during chromatin remodeling. *Mol Cell Biol* **22**:3255-63.
19. **Fryer, C. J., and T. K. Archer.** 1998. Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature* **393**:88-91.
20. **Fu, M., C. Wang, J. Wang, X. Zhang, T. Sakamaki, Y. G. Yeung, C. Chang, T. Hopp, S. A. Fuqua, E. Jaffray, R. T. Hay, J. J. Palvimo, O. A. Janne, and R. G. Pestell.** 2002. Androgen receptor acetylation governs trans activation and MEKK1-induced apoptosis without affecting in vitro sumoylation and trans-repression function. *Mol Cell Biol* **22**:3373-88.
21. **Fujimoto, N., A. Mizokami, S. Harada, and T. Matsumoto.** 2001. Different expression of androgen receptor coactivators in human prostate. *Urology* **58**:289-94.
22. **Fujimoto, N., S. Yeh, H. Y. Kang, S. Inui, H. C. Chang, A. Mizokami, and C. Chang.** 1999. Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J Biol Chem* **274**:8316-21.
23. **Gelmann, E. P.** 2002. Molecular biology of the androgen receptor. *J Clin Oncol* **20**:3001-15.
24. **Gregory, C. W., B. He, R. T. Johnson, O. H. Ford, J. L. Mohler, F. S. French, and E. M. Wilson.** 2001. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res* **61**:4315-9.
25. **Guan, K. L., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* **192**:262-7.
26. **Hayward, S. W., M. A. Rosen, and G. R. Cunha.** 1997. Stromal-epithelial interactions in the normal and neoplastic prostate. *Br J Urol* **79 Suppl 2**:18-26.
27. **Heinlein, C. A., and C. Chang.** 2002. Androgen receptor (AR) coregulators: an overview. *Endocr Rev* **23**:175-200.
28. **Hsiao, P. W., C. J. Fryer, K. W. Trotter, W. Wang, and T. K. Archer.** 2003. BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. *Mol Cell Biol* **23**:6210-20.
29. **Hu, Y. C., S. Yeh, S. D. Yeh, E. R. Sampson, J. Huang, P. Li, C. L. Hsu, H. J. Ting, H. K. Lin, L. Wang, E. Kim, J. Ni, and C. Chang.** 2004. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. *J Biol Chem* **279**:33438-46.
30. **Huang, Z. Q., J. Li, L. M. Sachs, P. A. Cole, and J. Wong.** 2003. A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. *Embo J* **22**:2146-55.
31. **Ichinose, H., J. M. Garnier, P. Chambon, and R. Losson.** 1997. Ligand-dependent interaction between the estrogen receptor and the human homologues of SWI2/SNF2. *Gene* **188**:95-100.

32. **Inoue, H., T. Furukawa, S. Giannakopoulos, S. Zhou, D. S. King, and N. Tanese.** 2002. Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. *J Biol Chem* **277**:41674-85.
33. **Ito, T., M. Yamauchi, M. Nishina, N. Yamamichi, T. Mizutani, M. Ui, M. Murakami, and H. Iba.** 2001. Identification of SWI.SNF complex subunit BAF60a as a determinant of the transactivation potential of Fos/Jun dimers. *J Biol Chem* **276**:2852-7.
34. **Jenster, G.** 1999. The role of the androgen receptor in the development and progression of prostate cancer. *Semin Oncol* **26**:407-21.
35. **Kadam, S., and B. M. Emerson.** 2003. Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. *Mol Cell* **11**:377-89.
36. **Kang, H., K. Cui, and K. Zhao.** 2004. BRG1 controls the activity of the retinoblastoma protein via regulation of p21CIP1/WAF1/SDI. *Mol Cell Biol* **24**:1188-99.
37. **Kim, I. Y., J. H. Kim, D. J. Zelner, H. J. Ahn, J. A. Sensibar, and C. Lee.** 1996. Transforming growth factor-beta1 is a mediator of androgen-regulated growth arrest in an androgen-responsive prostatic cancer cell line, LNCaP. *Endocrinology* **137**:991-9.
38. **Kinyamu, H. K., and T. K. Archer.** 2004. Modifying chromatin to permit steroid hormone receptor-dependent transcription. *Biochim Biophys Acta* **1677**:30-45.
39. **Kitagawa, H., R. Fujiki, K. Yoshimura, Y. Mezaki, Y. Uematsu, D. Matsui, S. Ogawa, K. Unno, M. Okubo, A. Tokita, T. Nakagawa, T. Ito, Y. Ishimi, H. Nagasawa, T. Matsumoto, J. Yanagisawa, and S. Kato.** 2003. The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113**:905-17.
40. **Knudsen, E. S., and J. Y. Wang.** 1997. Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol Cell Biol* **17**:5771-83.
41. **Knudsen, K. E., K. C. Arden, and W. K. Cavenee.** 1998. Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells. *J Biol Chem* **273**:20213-22.
42. **Kuo, M. H., and C. D. Allis.** 1998. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**:615-26.
43. **Marshall, T. W., K. A. Link, C. E. Petre-Draviam, and K. E. Knudsen.** 2003. Differential requirement of SWI/SNF for androgen receptor activity. *J Biol Chem* **278**:30605-13.
44. **Miyamoto, H., S. Yeh, G. Wilding, and C. Chang.** 1998. Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. *Proc Natl Acad Sci U S A* **95**:7379-84.
45. **Muchardt, C., and M. Yaniv.** 1993. A human homologue of *Saccharomyces cerevisiae* SNF2/SWI2 and *Drosophila* brm genes potentiates transcriptional activation by the glucocorticoid receptor. *Embo J* **12**:4279-90.
46. **Narlikar, G. J., H. Y. Fan, and R. E. Kingston.** 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**:475-87.
47. **Nie, Z., Y. Xue, D. Yang, S. Zhou, B. J. Deroo, T. K. Archer, and W. Wang.** 2000. A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. *Mol Cell Biol* **20**:8879-88.
48. **Oruetxebarria, I., F. Venturini, T. Kekalainen, A. Houweling, L. M. Zuijderduijn, A. Mohd-Sarip, R. G. Vries, R. C. Hoeben, and C. P. Verrijzer.** 2004. P16INK4a is

- required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. *J Biol Chem* **279**:3807-16.
49. **Palvimo, J. J., P. J. Kallio, T. Ikonen, M. Mehto, and O. A. Janne.** 1993. Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. *Mol Endocrinol* **7**:1399-407.
 50. **Peterson, C. L., and J. L. Workman.** 2000. Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr Opin Genet Dev* **10**:187-92.
 51. **Poukka, H., P. Aarnisalo, U. Karvonen, J. J. Palvimo, and O. A. Janne.** 1999. Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J Biol Chem* **274**:19441-6.
 52. **Quigley, C. A., A. De Bellis, K. B. Marschke, M. K. el-Awady, E. M. Wilson, and F. S. French.** 1995. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev* **16**:271-321.
 53. **Reisman, D. N., J. Sciariotta, T. W. Bouldin, B. E. Weissman, and W. Funkhouser, Jr.** 2004. The expression of the SWI/SNF ATPase subunits BRG1 and BRM in normal human tissues. *Applied Immunohistochemistry and Molecular Morphology* **in press**.
 54. **Reisman, D. N., M. W. Strobeck, B. L. Betz, J. Sciariotta, W. Funkhouser, Jr., C. Murchardt, M. Yaniv, L. S. Sherman, E. S. Knudsen, and B. E. Weissman.** 2002. Concomitant down-regulation of BRM and BRG1 in human tumor cell lines: differential effects on RB-mediated growth arrest vs CD44 expression. *Oncogene* **21**:1196-207.
 55. **Reyes, J. C., J. Barra, C. Muchardt, A. Camus, C. Babinet, and M. Yaniv.** 1998. Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). *Embo J* **17**:6979-91.
 56. **Roberts, C. W., M. M. Leroux, M. D. Fleming, and S. H. Orkin.** 2002. Highly penetrant, rapid tumorigenesis through conditional inversion of the tumor suppressor gene *Snf5*. *Cancer Cell* **2**:415-25.
 57. **Rouleau, N., A. Domans'kyi, M. Reebe, A. M. Moilanen, K. Havas, Z. Kang, T. Owen-Hughes, J. J. Palvimo, and O. A. Janne.** 2002. Novel ATPase of SNF2-like protein family interacts with androgen receptor and modulates androgen-dependent transcription. *Mol Biol Cell* **13**:2106-19.
 58. **Rowan, B. G., N. Garrison, N. L. Weigel, and B. W. O'Malley.** 2000. 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. *Mol Cell Biol* **20**:8720-30.
 59. **Shang, Y., M. Myers, and M. Brown.** 2002. Formation of the androgen receptor transcription complex. *Mol Cell* **9**:601-10.
 60. **Siddiqui, H., D. A. Solomon, R. W. Gunawardena, Y. Wang, and E. S. Knudsen.** 2003. Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition. *Mol Cell Biol* **23**:7719-31.
 61. **Sitz, J. H., M. Tigges, K. Baumgartel, L. G. Khaspekov, and B. Lutz.** 2004. Dyrk1A potentiates steroid hormone-induced transcription via the chromatin remodeling factor Arip4. *Mol Cell Biol* **24**:5821-34.
 62. **Sommer, S., and S. A. Fuqua.** 2001. Estrogen receptor and breast cancer. *Semin Cancer Biol* **11**:339-52.

63. **Spencer, T. E., G. Jenster, M. M. Burcin, C. D. Allis, J. Zhou, C. A. Mizzen, N. J. McKenna, S. A. Onate, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* **389**:194-8.
64. **Sterner, D. E., and S. L. Berger.** 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* **64**:435-59.
65. **Strobeck, M. W., K. E. Knudsen, A. F. Fribourg, M. F. DeCristofaro, B. E. Weissman, A. N. Imbalzano, and E. S. Knudsen.** 2000. BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci U S A* **97**:7748-53.
66. **Strobeck, M. W., D. N. Reisman, R. W. Gunawardena, B. L. Betz, S. P. Angus, K. E. Knudsen, T. F. Kowalik, B. E. Weissman, and E. S. Knudsen.** 2002. Compensation of BRG-1 function by Brm: insight into the role of the core SWI-SNF subunits in retinoblastoma tumor suppressor signaling. *J Biol Chem* **277**:4782-9.
67. **Torchia, J., C. Glass, and M. G. Rosenfeld.** 1998. Co-activators and co-repressors in the integration of transcriptional responses. *Curr Opin Cell Biol* **10**:373-83.
68. **Urnov, F. D., and A. P. Wolffe.** 2001. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). *Oncogene* **20**:2991-3006.
69. **Versteeg, I., N. Sevenet, J. Lange, M. F. Rousseau-Merck, P. Ambros, R. Handgretinger, A. Aurias, and O. Delattre.** 1998. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature* **394**:203-6.
70. **Vignali, M., A. H. Hassan, K. E. Neely, and J. L. Workman.** 2000. ATP-dependent chromatin-remodeling complexes. *Mol Cell Biol* **20**:1899-910.
71. **Wang, W., T. Chi, Y. Xue, S. Zhou, A. Kuo, and G. R. Crabtree.** 1998. Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes. *Proc Natl Acad Sci U S A* **95**:492-8.
72. **Wang, W., J. Cote, Y. Xue, S. Zhou, P. A. Khavari, S. R. Biggar, C. Muchardt, G. V. Kalpana, S. P. Goff, M. Yaniv, J. L. Workman, and G. R. Crabtree.** 1996. Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. *Embo J* **15**:5370-82.
73. **Yeh, S., and C. Chang.** 1996. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc Natl Acad Sci U S A* **93**:5517-21.
74. **Yoshinaga, S. K., C. L. Peterson, I. Herskowitz, and K. R. Yamamoto.** 1992. Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. *Science* **258**:1598-604.
75. **Zhang, H. S., A. A. Postigo, and D. C. Dean.** 1999. Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition. *Cell* **97**:53-61.
76. **Zhang, J., T. Z. Thomas, S. Kasper, and R. J. Matusik.** 2000. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. *Endocrinology* **141**:4698-710.

FIGURE LEGENDS

FIG. 1. BAF57 is expressed in the luminal epithelia. Mouse prostate was resected and the individual lobes [ventral lobe (VL, lane 3), dorsal-lateral lobe (DLL, lane 4), and anterior lobe (AL, lane 5)] were isolated and prepared for either immunoblotting or immunohistochemistry, as described. (A) BAF57 and AR were detected by immunoblot. LNCaP cell lysate (lane 1) and BT549 cell lysate (lane 2) were used as positive and negative controls, respectively. (B) Tissue from mouse prostate was sectioned and stained using standard immunohistochemistry techniques with no primary antibody control (left panels) or BAF57 (right panels). Antibody reactivity was visualized with DAB chromogen and nuclei were counterstained with hematoxylin. Top panels are 20X magnification, and bottom panels are 40X magnification.

FIG. 2. BAF57 binds the androgen receptor independent of ligand. (A) Immobilized GST or GST-BAF57 was incubated with [35 S]-methionine labeled AR in the presence of 0.1% EtOH vehicle (lanes 2, 3) or 10nM DHT (lanes 4, 5) for 3 hours at 4°C, washed, and denatured. Input and bound protein was subjected to SDS-PAGE and [35 S]-AR detected by autoradiography. (B) Clarified protein lysates from LNCaP cells were utilized for co-immunoprecipitation with antibodies against AR (lane 1), BAF57c (specific to C-terminus of BAF57) (71) (lane 2), or pre-immune serum (lane 3). Resultant immunoprecipitates were subjected to SDS-PAGE and the presence of AR was detected by immunoblotting.

FIG. 3. BAF57 is critical for androgen receptor activity. (A) MCF7 (lane 1), BT549 (lane 2), LNCaP (lane 3), and CV1 (lane 4) cells were harvested, lysed, and subjected to SDS-PAGE. Immunoblots were performed with antibodies against BAF57 and CDK4 (loading control). (B) MCF7, CV1, and BT549 cells (2×10^5) were transfected with 0.5 μ g pARR2-Luciferase reporter, 0.5 μ g pSG5AR, and 0.25 μ g pTK-renilla luciferase (to normalize for transfection efficiency).

Post transfection, cells were stimulated with either 0.1% EtOH vehicle or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and monitored for luciferase activity utilizing the dual Luciferase Assay Reporter System. AR activity is presented as fold activation by ligand (DHT) over EtOH. Averages and standard deviations are shown. (C) BT549 cells either untransfected (lane 1) or transfected as above with pGreenLantern (indicates transfection) and pSG5AR (lane 2) were harvested 24 hours post-transfection. Immunoblot analysis demonstrates AR expression in transfected BT549 cells. (D) To elucidate BAF57 function on AR reporter activity, BT549 cells (2×10^5) were transfected with 0.5 μ g pARR2-Luciferase reporter, 0.5 μ g pSG5AR, 0.25 μ g pTK-renilla luciferase, and either 2.25 μ g parental vector or pBabe-WTBAF57. Post transfection, cells were treated with either 0.1% EtOH vehicle or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and luciferase activity was measured using the dual Luciferase Assay Reporter System. Ligand induced AR activity in the presence of vector was set to “100,” and relative luciferase activity is shown. (E) BT549 cells (2×10^5) were transfected with 0.5 μ g pGreenLantern GFP (transfection control), 0.5 μ g SG5AR, and either 2.25 μ g of vector (lane 1) or BAF57 expression plasmid (lane 2). Immunoblotting was performed for AR and GFP at 24 hour post-transfection.

FIG. 4. Abrogation of BAF57 results in reduced endogenous androgen receptor activity.

(A) LNCaP cells (2×10^5) were transfected with 1 μ g pARR2-Luciferase reporter, 0.25 μ g pTK-renilla luciferase, and either 2.25 μ g pBabe-BAF57 Δ N (dominant negative BAF57) or parental vector (to 4.5 μ g total DNA). Cells were treated with either 0.1% EtOH vehicle or 1nM DHT for 48 hours (with re-stimulation after 24 hours), harvested, and lysed. Luciferase activity was measured as in Fig. 3D and AR activity in the presence of vector alone plus DHT was set to “100.” Average relative luciferase activity and standard deviations are shown (* p-value < 0.05). (B) LNCaP cells transfected with either vector (lane 1) or BAF57 Δ N (lane 2) were harvested and

the expression of endogenous AR and CDK4 (loading control) was determined by immunoblotting. (C) LNCaP cells (4.5×10^5) were transfected with $1 \mu\text{g}$ pBabe-PURO, $1 \mu\text{g}$ H2B-GFP, and $4 \mu\text{g}$ of parental vector (lane 1), BAF57 Δ N (lane 2), or dominant negative AR (dnAR) (lane 3) expression plasmids. Post transfection, cells were selected with puromycin until greater than 90% of cells were H2B-GFP positive (approximately 3 days). Cells were then harvested and RNA was isolated and subjected to RT-PCR. cDNAs were amplified in the presence of ^{32}P -dCTP to accurately quantify the signal (upper panel). Product levels were quantified using a Phosphor-Imager. Relative levels of PSA over GAPDH from two independent experiments and standard deviations are shown (lower panel).

FIG. 5. BAF57 action is dependent upon SWI/SNF ATPase activity. (A) BT549 cells (2×10^5) were transfected with $0.5 \mu\text{g}$ pARR2-Luciferase reporter, $0.25 \mu\text{g}$ pTK-renilla luciferase, $0.5 \mu\text{g}$ pSG5AR, and $2.25 \mu\text{g}$ pBabe-WTBAF57, $1 \mu\text{g}$ dominant negative BRM (dnBRM), or parental vector (to $4.5 \mu\text{g}$ total DNA) as indicated. Post transfection, cells were treated with 0.1% EtOH or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and dual luciferase activity was measured. Ligand activated AR activity was set to “100,” and relative luciferase activity and standard deviations are shown (* p-value < 0.05). (B) BT549 cells were transfected with $0.5 \mu\text{g}$ SG5AR, $0.5 \mu\text{g}$ pGreenLantern GFP, and $1 \mu\text{g}$ vector (lane 1) or dnBRM (lane 2) expression plasmid. Cell lysates were obtained and immunoblot analysis was performed for AR and GFP. (C) SW13 cells (2×10^5) were transfected with $0.5 \mu\text{g}$ pARR2-Luciferase reporter, $0.25 \mu\text{g}$ pTK-renilla luciferase, $0.5 \mu\text{g}$ pSG5AR, and $2.25 \mu\text{g}$ pBabe-WTBAF57, $1 \mu\text{g}$ BRM, and parental vector (to $4.5 \mu\text{g}$ total DNA) as indicated. Post transfection, cells were treated with 0.1% EtOH or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and dual luciferase activity was measured. Ligand activated AR activity was set to “100,” and relative luciferase activity and standard deviations are shown.

FIG. 6. BAF57 is recruited to the AR regulatory regions in the presence of ligand. LNCaP cells (5×10^6) were cultured in steroid free media for 4 days, then stimulated with either 0.1% EtOH vehicle (lane 1) or 10nM DHT (lane 2) for 60 min. Post treatment, cells were formaldehyde cross-linked and chromatin was recovered for use in ChIP assays. (A, B) Antibodies to acetylated histone H4 (AcH4), AR, BAF57h and pre-immune serum were used for ChIP analyses of the PSA enhancer region, as indicated. (C) 1×10^5 LB1-Luc cells (with integrated pARR2-Luc) were serum starved for 3 days, then treated with the indicated concentrations of DHT for 24 hours. Reporter analysis was performed and demonstrated activation of the AR in a dose dependent manner (left panel). (Right panel, ChIP analysis) LB1-Luc cells (5×10^6) were cultured in steroid free media for 4 days, then stimulated with either 0.1% EtOH vehicle (lane 1) or 10nM DHT (lane 2) for 60 min. Post treatment, cells were formaldehyde cross-linked and chromatin was recovered for use in ChIP assays. Antibodies to AR and BAF57c were used for ChIP analyses of the ARR2 promoter region.

FIG. 7. BAF57 cooperates with p160 co-activators. BT549 cells (2×10^5) were transfected with 0.5 μ g pARR2-Luciferase reporter, 0.25 μ g pTK-renilla luciferase, 0.5 μ g pSG5AR, and 2.25 μ g pBabe-WTBAF57, 1 μ g of co-activator [pRC3.1-SRC1 (A) or pCMV-TIF2 (B)], or parental vector (to 4.5 μ g total DNA) as indicated. Post transfection, cells were treated with 0.1% EtOH or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and dual luciferase activity was measured. Ligand activated AR activity was set to “100,” and average relative luciferase activity and standard deviations are shown.

FIG. 8. BAF57 facilitates non-p160 androgen receptor co-activator function. (A) BT549 cells (2×10^5) were transfected with 0.5 μ g pARR2-Luciferase reporter, 0.25 μ g pTK-renilla

luciferase, 0.5 μ g pSG5AR, and 0.01-2.25 μ g pBabe-WTBAF57 in the absence (black bars) or presence of 1 μ g pSG5ARA70 (stippled bars). As described in the Materials and Methods section, all transfections were brought to a total 4.5 μ g using empty vector. Post transfection, cells were treated with 0.1% EtOH or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and luciferase activity was measured. Ligand activated AR activity was set to “100” (data not shown) and average relative luciferase activity and standard deviations are shown ($p < 0.05$). (B-D) BT549 cells (2×10^5) were transfected with 0.5 μ g pARR2-Luciferase reporter, 0.25 μ g pTK-renilla luciferase, 0.5 μ g pSG5AR, and 2.25 μ g pBabe-WTBAF57, vector, or the indicated co-activator (pSG5ARA55, pFLAG-Ubc9) or mutant (pcDNA3-ARA70N2) expression plasmid (1 μ g). Post transfection, cells were treated with 0.1% EtOH or 0.1nM DHT for 24 hours. Cells were harvested, lysed, and luciferase activity was measured. Ligand activated AR activity in the absence of BAF57 was set to “100,” and average relative luciferase activity and standard deviations are shown ($p < 0.05$).

FIG. 9. Abrogation of BAF57 or BRM attenuates androgen receptor dependent proliferation. (A) LNCaP cells (1×10^5) were transfected with 0.5 μ g pEGFP-H2B-GFP and 4.5 μ g of parental vector, pBabe-BAF57 Δ N, or dominant negative AR (pSG5AR- Δ 46-408). Two days post transfection, cells were labeled with BrdU for 16 hours and fixed. The percent of transfected cells (H2B-GFP positive) incorporating BrdU was determined (left panel, representative image). The effect of dnAR or BAF57 Δ N is shown as percent inhibition of BrdU incorporation over vector control (right panel). (B) LNCaP cells were transfected with 0.5 μ g H2B-GFP and 4.5 μ g of either pHTP-vector (lane 2) or pHTP-BRMi (lane 3). Post transfection, cells were selected using puromycin. Once cells were greater than 90% H2B-GFP positive they were lysed and subjected to immunoblot analyses for BRM and actin (loading control). SW13 cells (lane 1) are shown as a positive control for BRM deficiency. (C) LNCaP cells were

transfected with 0.5 μ g H2B-GFP and 4.5 μ g parental vector, pHTP-BRMI scrambled, pHTP-BRMI, or pCMV-p16ink4a. The percent of transfected cells (H2B-GFP positive) incorporating BrdU was determined. The effect of the BRM attenuation is shown as percent inhibition of BrdU incorporation over vector control.

Figure 1

Figure 2

Figure 3

Figure 4**A**

LNCaP

B **C**

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Chapter IV

Targeting the BAF57 SWI/SNF Subunit in Prostate Cancer: a novel platform to control AR activity

Kevin A. Link,¹ Clay E. Comstock,¹ Sucharitha Balasubramaniam,¹ Nathan Powers,¹ Khanh H. Cao,¹ Annemie Haelens,⁴ Frank Claessens,⁴ Monica P. Revelo,⁵ Karen E. Knudsen^{1,2,3*}

Department of Cell Biology,¹ UC Barrett Cancer Center,² and Center for Environmental Genetics,³ University of Cincinnati College of Medicine, Cincinnati, Ohio, Department of Molecular Cell Biology, University of Leuven, Leuven, Belgium,⁴ and Department of Pathology, University of Utah, Salt Lake City, Utah⁵

*Corresponding author. Mailing address: Department of Cell Biology, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521. Phone: (513) 558-7371. Fax: (513) 558-4454. E-mail: Karen.Knudsen@uc.edu

Abstract

Prostate cancer development and progression relies on the activity of the androgen receptor (AR). Therefore, there is a significant need to develop mechanisms that thwart AR function. It has been previously shown that upon ligand (androgen) binding, AR requires the action of the SWI/SNF chromatin remodeling complex to initiate target gene activation, and the non-essential SWI/SNF subunit BAF57 was identified as a potential mediator of this interaction. The present study was initiated to assess this hypothesis, and to challenge the consequence of targeting the AR-BAF57 interaction in prostate cancer. Examination of normal and tumor samples revealed that BAF57 expression is maintained and often enhanced in prostate cancers, indicating that, like AR, this potential target is prevalent in this tumor type. Subsequent investigation into the specificity of the BAF57 requirement revealed that the protein is recruited early to sites of AR activity after androgen stimulation. By contrast, BAF57 (or SWI/SNF function in general) is dispensable for antagonist action. Initial biochemical studies revealed that BAF57 specifically docks into the AR C-terminal extension (CTE), which resides within the AR hinge domain. This observation is significant, as this region of AR is not currently targeted during prostate cancer therapy. However, the ability to block AR association was developed through mapping of the AR association motif within BAF57. It was shown that a specific N-terminal region of BAF57 is sufficient for AR association. Importantly, introduction of this motif significantly disrupted AR occupancy at target genes, inhibited AR activity, and suppressed prostate cancer cell proliferation. Together, these observations

reveal the specificity of the BAF57 requirement, identify the biochemical mechanisms that support the AR-BAF57 association, and delineate the utility of targeting this interaction as a novel means to inhibit AR function in prostate cancer.

Introduction

The androgen receptor (AR) is a ligand activated transcription factor required for the development and progression of prostate cancer (12). AR is activated through binding of the high affinity ligand dihydrotestosterone (DHT) to the receptor C-terminal ligand binding domain (LBD) (4). Upon binding, AR is released from heat shock proteins, forms a homodimer, and translocates to the nucleus. In the nucleus, AR utilizes a highly conserved zinc finger DNA binding domain (DBD) and C-terminal extension (CTE, within the hinge region of AR) to permit binding to androgen responsive elements (AREs) often located within the promoter and/or enhancer regions of AR target genes, such as the prostate specific antigen (PSA) (9, 19, 42). AR binding to DNA initiates the recruitment of cofactors (such as the p160 class of co-activators and the SWI/SNF chromatin remodeling complex) that assist in inducing transcriptional activation and are necessary for generating an open chromatin structure (17). This open chromatin state permits subsequent binding of the basal transcription machinery to the promoter region and initiates transcription of the target gene through the actions of RNA polymerase II (21, 35, 47).

Given the dependence of prostate cancer on AR action, current treatment options are primarily focused on inhibiting the AR signaling pathway (2, 27, 41). Initial treatment of non-organ confined prostate cancer generally consists of agents which deplete AR ligand synthesis and/or act as direct AR antagonists that exclusively target the AR LBD. Whereas ligand depletion prevents AR activation and nuclear translocation, binding of the most commonly utilized

antagonist, bicalutamide (Casodex), to the LBD induces nuclear translocation and ARE binding; however, the antagonist induces co-repressor recruitment to the AREs, thus enforcing active repression of gene transcription (43, 57). The repressive action of bicalutamide has also been demonstrated to prevent AR NH₂/COOH-terminal interaction, which is required for AR activity in the context of chromatin (22, 33). At the cellular level, these combined mechanisms of AR inhibition (ligand depletion and antagonist administration) induce cell cycle arrest and/or cell death (7). Unfortunately, tumor cells circumvent these regimens by restoring AR activity, as achieved through: amplification and/or overexpression of AR, gain-of-function AR mutations, co-activator overexpression, or ligand independent AR activation (12, 46). Concordantly, it is well appreciated that AR remains the central therapeutic target for both early and late stage disease, and additional mechanisms must be developed to block AR function. Given the reliance of AR on cofactor recruitment outside the LBD, manipulation of this requirement may provide a cooperative mechanism to suppress AR function.

The SWI/SNF chromatin remodeling complex is a critical component for AR mediated gene transcription and resultant AR-dependent prostate cancer cell proliferation (25, 26, 31, 32). SWI/SNF consists of a core ATPase (either BRM or BRG1) and 8 to 10 accessory subunits termed BRG1-associated factors (BAFs). Conventionally, biochemical composition of the complex is thought to underlie the specificity of SWI/SNF activity. With regard to AR, the BRM ATPase is preferentially utilized for AR activation in response to agonist, whereas BRG1 is poorly supportive of AR activity in the model systems tested (26, 32). These

findings indicate that only a subset of SWI/SNF complexes is likely utilized by AR in response to androgen binding. Specificity of SWI/SNF function has also been demonstrated by examination of the BAF subunits, especially in regard to the regulation of nuclear receptors. BAF60a was suggested to serve as a bridging factor between the central BRG1 ATPase and the glucocorticoid receptor (GR) (24), whereas BAF250 was shown to serve as a specificity subunit for GR activation (36). Furthermore, BAF60c was demonstrated to enhance retinoic acid receptor (RAR), estrogen receptor (ER), and GR activity with an observed increase in RAR activity upon addition of p160 co-activators (13), demonstrating the versatility of the complex. By contrast, the BAF57 subunit was shown to directly bind AR and facilitate AR activity, whereas no association was observed between AR and the BRM ATPase (31). This observation is of significance, as BAF57 is not required for SWI/SNF function (37); rather, this non-classical subunit is proposed to bridge SWI/SNF to transcription factors and thereby selectively regulate transcription factor action. Functional investigations revealed that cells devoid of BAF57 are unable to support AR activity, and that re-introduction of the BAF57 subunit could rescue AR function only in the presence of an active BRM ATPase (31). The importance of BAF57 function was also established in the presence of AR co-activators that either contain or recruit histone acetyltransferases, wherein BAF57 cooperated with these cofactors to enhance AR activity. From these data it was predicted that BAF57 influences AR transcriptional activity through SWI/SNF. Consistent with this hypothesis, disruption of BAF57 activity was sufficient to suppress AR-dependent cellular

outcomes in prostate cancer cells. Given the requirement of BAF57 for AR action, dissection of the mechanisms that govern BAF57 function may be of critical importance for identifying new points of intervention in prostate cancer.

The present study discerns the mechanism of BAF57 recruitment, specificity of action, and establishes the consequence of this event for AR dependent prostate cancer cell proliferation. Initial studies revealed that like AR, BAF57 expression is maintained and/or elevated in prostatic adenocarcinoma of all grades, thus indicating that the protein likely remains important for AR signaling during cancer development and progression. The presence of BAF57 selectively influenced agonist function, as BAF57 and/or SWI/SNF activity proved dispensable for the transcriptional or proliferative response to antagonist. Given the influence of BAF57 on AR action, the mechanisms which underpin BAF57 recruitment to sites of AR action were subsequently delineated. BAF57 was shown to be recruited early after androgen stimulation to AREs, and docks with the AR through the DBD/hinge region. These findings indicate that BAF57 impinges on AR through domains not currently targeted in prostate cancer therapy and prompted subsequent studies to identify the AR binding motif within BAF57. Mapping studies revealed that BAF57 interacts with AR through the N-terminal domain, which is comprised of a proline rich (PR) region and a high mobility group (HMG) DNA binding domain. Remarkably, expression of the AR binding motif of BAF57 in prostate cancer cells was sufficient to reduce AR and BAF57 residence at AREs, inhibit AR-dependent transcription, and specifically suppress AR dependent proliferation. Together, these data identify the

mechanisms by which BAF57 regulates AR function, and demonstrate that abrogation of BAF57 recruitment may provide a new strategy to block AR function in prostate cancer.

Materials and Methods

Plasmids and reagents. The plasmids pSG5AR, pBabe-wtBAF57-flag, pBabe-BAF57 Δ N-flag, pCMV-p16ink4a, pcDNA3, pGEX-KG GST, pGEX 3X GST-BAF57, and pHTP-Scrambled have been previously described. (31) GST-AR 553-635 and GST-AR 634-668 were a generous gift from Dr. S. Balk (56). GST-AR 1-173 and GST-AR 1-660 were received from Dr. E. Wilson (20) and GST-AR 505-676 was obtained from Dr. Z. Sun (45). pcDNA3.1(-) BAF57-flag, pcDNA3.1(-) BAF57 Δ PR-flag, and pcDNA3.1(-) BAF57 Δ HMG-flag were provided by Dr. T. Archer (5). pSG5AR Δ 629-636 was received from Dr. F. Claessens (18). pHTP-vector, pHTP-BRMi, and pHTP-BRG1i were gifts from Dr. Bernard Weissman (40). pcDNA3 BAF57 Δ N-flag was generated by subcloning BAF57-flag from the pBabe plasmid using BamHI and EcoRI restriction sites. pcDNA3.1 BAF57 1-145 3Xflag was generated by PCR amplification of the first 145 amino acids of BAF57 with the addition of a BamHI restriction site at the 3' end. PCR amplification was followed by TA cloning (Invitrogen). BAF57 1-145 was then cloned into the pcDNA3.1 3Xflag vector using the EcoRI and BamHI restriction sites. The construct was verified by sequencing. Dihydrotestosterone (DHT) was from Sigma. Casodex (bicalutamide) was obtained from AstraZeneca Pharmaceuticals (London, United Kingdom) and was used at 1 μ M for a 48h treatment period where indicated.

BAF57 antibody generation. BAF57 antibody generation was carried out through collaboration with Bethyl Laboratories (Montgomery, TX). Briefly, a 20 amino acid peptide sequence (amino acids 291-310) of BAF57 was synthesized,

purified by HPLC, and verified by mass spectrometry. The peptide was conjugated to KLH and rabbits were immunized. Anti-BAF57 was affinity purified from the rabbit serum.

Immunohistochemistry. Hematoxylin and Eosin (H&E) staining and IHC were performed as previously described (55). Briefly, tissue sections were treated with the Vectastain Elite ABC rabbit staining kit and developed for 2 min with the 3,3'-diaminobenzidine substrate kit according to manufacturer specifications (Vector Laboratories, Inc., Burlingame, CA). Specificity and optimal dilution of the rabbit polyclonal anti-BAF57 antibody (1:2000) was determined using tissue sections from cell culture pellets obtained from BT549 (BAF57-negative) and LNCaP (BAF57-positive) cells (protocol adapted from (10)). Cell culture pellets for IHC were generated by gently scraping asynchronously growing cultures in phosphate buffered saline and pelleted in a 15 ml conical tube. Cell pellets were suspended in 3 drops of human plasma and thrombin was added to produce a cell clot. The clot was suspended in 10 ml of 10% neutral buffered formalin for 24 hr, then embedded in paraffin and sectioned for analysis. Further validation of the anti-BAF57 antibody was determined using tissue sections from localized and lymph node metastatic prostate cancer specimens obtained from the University of Cincinnati Hospital in accordance with institutional review board standards. BAF57 expression was determined using a tissue microarray slide (TMA) containing 80 cores (PR801; US Biomax, ljamsvillve, MD). Patient tumors and the TMA were evaluated, graded, and semi-quantitatively scored by a pathologist (M. P. R.) according to established

guidelines (11). Immunoreactivity of BAF57 was scored on intensity: 0 (none), + (low), ++ (moderate), +++ (high) and extent of tumor staining: 0 (none), 1 (<25%), 2 (>25%, <50%), 3 (>50%). The final BAF57 IHC score is displayed as a composite (intensity + extent) (1).

ChIP. Time course ChIPs were performed as previously described in the LB1-luc cell line (31). Briefly, cells were seeded in phenol red free IMEM containing 5% CDT. After 72 hrs, cells were stimulated with either 0.1% EtOH or 10nM DHT for the indicated amount of time. Cells were cross-linked with 1% formaldehyde, then quenched with 0.125M glycine for quenching. Cells were washed in PBS and lysed in cell lysis buffer. Nuclei were then lysed in nuclear lysis buffer, sonicated, and clarified. Lysate was divided for each IP (AR and IgG or BAF57 and IgG) and was carried out in ~500μl total volume of RIPA. Following incubation for 1hr, protein A sepharose (GE Healthcare) was added and the IP was continued overnight. Beads were then washed 3x with super RIPA (RIPA buffer with 300mM NaCl), 1x with RIPA, and 1x with TE. ChIP extraction buffer was then added to sample, along with RNase A, and 20μl 5M NaCl. Cross-linking was reversed by incubation at 65°C for 4 hrs up to overnight. DNA was purified with the QIAquick PCR purification kit (Qiagen). PCR analysis was carried out to examine AR and BAF57 recruitment to the AREIII region within PSA using primers previously described (43). To examine AR and BAF57 recruitment in the presence and absence of BAF57 1-145, LNCaP cells were seeded at a density of 1×10^6 on poly-L-lysine coated 10cm dishes in IMEM containing 5% ΔFBS. Cells were transfected the following day using the lipofectin transfection reagent

(Invitrogen) with 2 μ g H2B-GFP and 8 μ g of either pcDNA3 vector or pcDNA3.1 BAF57 1-145 3Xflag. After 18 hours, the media was changed to IMEM containing 5% CDT. Forty-eight hours post transfection, cells were stimulated with 10nM DHT for 1hr and harvested as above.

BrdU incorporation. LNCaP cells were seeded on poly-L-lysine coated coverslips and transfected with Lipofectin (Invitrogen). Plasmid transfection consisted of H2B-GFP, and one of the following expression plasmids: pcDNA3 (vector control), pBabe-BAF57 Δ N-flag, pCMV-p16ink4a, dnAR (pSG5AR Δ 46-408), or pcDNA3 BAF57 1-145 3Xflag. Post-transfection, bromodeoxyuridine (BrdU; GE Healthcare) was added for a 16h incubation period. Indirect immunofluorescence was performed as previously described (29) to detect BrdU. H2B-GFP positive cells (marking transfection) were screened for BrdU positive staining. Results are plotted as percent BrdU incorporation (relative to vector) or as percent inhibition versus vector control, as indicated. Data shown are reflective of at least three independent experiments.

GST pull-down. Expression of indicated GST fusion proteins was obtained through induction by 0.1mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 3 to 5 h. Bacterial cells were collected, washed with STE (150mM NaCl, 25mM Tris [pH 8.0], and 1mM EDTA), and resuspended in 3ml of STE. Cell lysis was carried out with the addition of 0.1 mg/ml of lysozyme and 1.5% Sarkosyl followed by sonication. The clarified supernatant was then incubated with the appropriate amount of glutathione-agarose beads at 4 $^{\circ}$ overnight. Beads were washed four times in PBS and incubated overnight at 4 $^{\circ}$

with *in vitro* transcribed/translated [³⁵S]-methionine labeled protein (TnT-Coupled Reticulocyte Lysate system [Promega]). Reactions were carried out in 500μl NETN (20mM Tris-HCl [pH 8.0], 1mM EDTA, 100mM NaCl, 0.5% NP-40) supplemented with PMSF and protease inhibitors. Samples were separated on an SDS-PAGE, stained with Coomassie blue, and subjected to fluorography by using Fluoro-Hance (Research Products International Corp., Mount Prospect, Ill.). Labeled protein was detected by autoradiography and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Tissue culture and treatment. BT549 cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 2mM L-glutamine, and 100 U/ml of penicillin-streptomycin (Mediatech, Herndon, VA). For culture in steroid-free conditions, cells were cultured in phenol-red free RPMI and 10% charcoal dextran-treated FBS (CDT; Atlanta Biologicals, Norcross, GA). HEK293 cells were maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine, and 100 U/ml of penicillin-streptavidin.

Reverse Transcriptase-PCR. LNCaP cells were seeded at a density of 4×10^5 in poly-L-lysine coated 6cm dishes and transfected overnight with the Lipofectin transfection reagent (Invitrogen) with 1μg pEGFP H2B-GFP to monitor for transfection efficiency, 1μg pBabe-PURO for rapid puromycin selection, and 4μg of one of the following expression plasmids: pcDNA3 (vector control), pHTP (sivector control), pHTP-BRMi, pHTP-BRG1i, pBabe-BAF57ΔN, pSG5ARΔ46-408 (positive control), or pcDNA3.1 BAF57 1-145 3Xflag. After transfection, cells

were allowed to recover for approximately 24 hours. Transfected cells were selected using 0.625 μ g/mL puromycin (Sigma). Selection was carried out until greater than 90% of cells were H2B-GFP positive (approximately 2 days). RNA was isolated using the TRIzol Reagent (Gibco) as recommended by the manufacturer. To quantify expression, RT-PCR was performed with amplification of the cDNA. Primers were used for prostate specific antigen (PSA) (primer pair: 5'-CTTGTAGCCTCTCGTGGCAG-3'; 5'-GACCTTCATAGCATCCGT GAG-3'), TMPRSS2 (primer pair 5'-AATCGGTGTGTTGCGCCTCTAC-3'; 5'-GCGGCT GTCACGATCC-3'); and GAPDH (loading control) (primer pair: 5'-CCACCCCATGGCAAATTCCATGGCA-3'; 5'-TCTAGACGGCAGGTCAGGTCC ACC-3'). PCR conditions were as follows: PSA and GAPDH 94°C for 2 min.; 21 cycles (PSA) or 28 cycles (GAPDH) of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and 72°C for 10 min. TMPRSS2 94°C 5 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and 72°C for 5 min. PCR reactions were separated on a 1% agarose gel and band intensity was determined. For comparison, AR target gene expression (PSA or TMPRSS2/GAPDH) in the presence of vector was set to 1. Representative data from at least two independent experiments is shown.

Immunoblotting. Cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail and phenylmethylsulfonylfluoride (PMSF). After brief sonication, the supernatant was clarified and protein quantitation was carried out using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Equal protein was separated

by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Membranes were immunoblotted with the indicated antibodies using standard techniques and visualized by chemiluminescence (Western-Lightning; Perkin-Elmer Life Sciences). Antibodies used were generated against BAF57 (amino acids 291-310 by Bethyl Laboratories; Montgomery, TX), lamin B (Santa Cruz), AR (N-20; Santa Cruz), CDK4 (H-22; Santa Cruz), and FLAG (Sigma).

Transfection and reporter analysis. BT549 cells were seeded in 12-well dishes (1×10^5 cells) or 6-well dishes (2×10^5 cells) in RPMI containing 10% CDT. Cells were transfected the following day with FuGENE6 transfection reagent (Roche Molecular Biochemicals). Transfections for BAF57 activation of wild type AR vs. AR Δ 629-636 consisted of 0.5 μ g pSG5AR or pSG5AR Δ 629-636, 0.5 μ g pARR2-Luc, 0.25 μ g pTK-Renilla-Luc (transfection control), and 2.25 μ g pBabe-wtBAF57-flag as indicated. Total plasmid DNA concentration was brought to 4.5 μ g with pcDNA3 empty vector. Following transfection, media was changed and 0.1nM DHT or 0.1% EtOH vehicle was added as indicated. Cells were treated for 24 hrs, harvested and lysed. Reporter analysis was carried out using the Dual Luciferase Assay Reporter System (Promega). Transfection for the impact of 1-145 on BAF57 activation of AR was carried out in 12-well dishes (1×10^5) cells. Transfection consisted of 0.25 μ g pSG5AR, 0.25 μ g pARR2-Luc, 0.125 μ g pTK-Renilla-Luc (transfection control), 2.25 μ g pBabe-wtBAF57-flag, and/or 0.5 μ g, 1.125 μ g, or 2.25 μ g (no wtBAF57) of 1-145. Treatment and reporter analyses were performed as above.

Coimmunoprecipitation. HEK293 cells were seeded at a density of 3×10^5 in 6cm dishes and transfected using the BBS-calcium phosphate protocol. Transfection consisted of 1 μ g H2B-GFP (to monitor transfection efficiency) and either pcDNA3 empty vector, pcDNA3.1(-) BAF57-flag, pcDNA3 BAF57 Δ N-flag, pcDNA3.1(-) BAF57 Δ PR-flag, or pcDNA3.1(-) BAF57 Δ HMG-flag. Following transfection, cells were harvested and lysed in NETN plus a protease inhibitor cocktail. After sonication and clarification, protein was quantified using the Bio-Rad DC protein assay kit. An equal concentration of protein was utilized in each IP. IPs were performed with the M2-flag resin (Sigma) in NETN for a period of 2hrs up to overnight at 4°C. Beads were then washed 4X in NETN, resuspended in 2X SDS-PAGE buffer, and boiled to denature proteins. Samples were then subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies.

Results

BAF57 expression is maintained and/or elevated in human prostate cancer specimens. To assess the impact of BAF57 on AR signaling in prostate cancer, BAF57 expression patterns were first examined in prostate cancer specimens. For these studies, antisera directed against BAF57 were generated as described in the Material and Methods section. Specificity of the antibodies was demonstrated by immunoblot (Fig. 1A). As expected, no immunoreactivity was detected in BT549 cells (lane 1), which are devoid of this SWI/SNF subunit (8). By contrast, BAF57 was readily apparent in LNCaP (prostatic adenocarcinoma) cells (lane 2) consistent with previous reports (31). Screening of additional prostate cancer cell lines (lanes 3-6) demonstrated that BAF57 was detected in all tested model systems. While these data were suggestive that BAF57 expression is retained in prostate cancer, immunohistochemical (IHC) studies were undertaken to quantify expression in primary tumors. To validate specificity of the BAF57 antibody for IHC, tissue sections from paraffin embedded BT549 and LNCaP cells were utilized (Fig. 1B). There was no detectable staining by IHC within the BT549 tissue sample (left panel). Comparatively, the LNCaP tissue showed significant expression of BAF57, with the expected nuclear staining (right panel), thus demonstrating the efficacy of the reagent for this approach. Subsequently, expression of BAF57 in non-neoplastic and primary prostate cancer specimens was examined (Fig. 1C). As expected, no positive staining was observed in the absence of the primary antibody (top panel). However, significant nuclear expression of BAF57 was evident in the presence of

primary antibody for the non-neoplastic sample (middle panel), with the majority of protein localized within the epithelial layer of the prostate glands and minimal expression observed in the stromal layer of the tissue. High nuclear BAF57 expression was also observed in the prostate cancer sample (bottom panel). Furthermore, analysis of BAF57 expression was carried out on two independent metastatic samples of prostate cancer (Fig. 1D). AR staining was used to validate the specimen as prostate cancer (middle panels) and was highly expressed, as expected; the H & E control sections were also shown (left panels). Similarly, BAF57 was highly expressed in the two tissue samples examined (right panels). Thus, BAF57 expression can be clearly observed in both localized and metastatic prostate cancers.

To validate these findings in a larger context, a tissue microarray (TMA) containing both tumor tissue and non-neoplastic controls was stained and scored as previously described (1) (Fig. 1E). Metastatic tissue samples were also included in the scoring process, and the “n” number is provided above each data point. All non-neoplastic prostate tissue scored positive for BAF57 staining as expected based on the requirement of AR function for the survival of these cells (46). As shown, tumors of all Gleason grades scored positive for BAF57, with Gleason 6-8 and metastatic samples showing a trend toward increased BAF57 expression. Although these differences did not reach statistical significance, these data demonstrate that BAF57 expression is maintained in prostate cancer, and suggest that a subset of tumors may harbor elevated BAF57 expression. Congruent with this supposition, microarray profiling of BAF57 mRNA expression

in metastatic prostate cancer (as obtained in the Oncomine database (39)) revealed a similar trend of elevated BAF57 expression in tumor tissue (Supplemental Fig. 1) (30, 48). Together, these data indicate that BAF57 expression is retained and/or elevated in prostate cancer, and therefore may provide an additional therapeutic target by which to deplete AR activity. To test this hypothesis, it was necessary to first dissect the mechanisms by which BAF57 regulates AR.

BAF57 is temporally recruited to the PSA enhancer element. The recruitment of AR and BAF57 to multiple androgen responsive elements (AREs) in response to ligand has previously been established (31, 43). Given the current evidence of intermittent signaling and/or temporal recruitment of many co-activating factors (34), the kinetics of AR and BAF57 recruitment to the PSA locus was determined through chromatin immunoprecipitation analyses (ChIP) (Fig. 2). AR was continually detected over a five hour period (A), consistent with previous reports (52). However, BAF57 recruitment differed from the temporal association of AR over this period. While BAF57 was recruited after 1hr stimulation with dihydrotestosterone (DHT) and appeared to be maintained until three hours post-treatment, the protein was not detected at the PSA locus during subsequent timepoints. Thus, these data indicate that BAF57 action at the ARE occurs early after agonist stimulation, concurrent with the requirement of AR for chromatin remodeling activity.

SWI/SNF ATPases and BAF57 are not required for bicalutamide mediated repression of AR activity. As SWI/SNF is needed for AR function,

and the subunit that mediates AR activity is expressed at high levels in prostate cancer, it could be hypothesized that blocking SWI/SNF recruitment may provide a new mechanism to suppress AR activity in cancer. However, the importance of SWI/SNF for the response to AR antagonist used in therapy has not heretofore been assessed. To address this, the impact of SWI/SNF was initially examined after knockdown of the central ATPases, BRM (60% knockdown) or BRG1 (40% knockdown) (Fig. 3A, left panel). LNCaP cells were transfected with H2B-GFP and either vector, BRMi, or BRG1i and treated with bicalutamide as indicated (Fig. 3A, right panel). As expected, the AR target gene PSA was highly expressed in the vector transfected, vehicle treated sample (lane 1). Upon treatment with bicalutamide, relative expression of PSA was reduced by 49% as compared to vehicle treated (lane 2 vs. lane 1). Additionally, knockdown of BRM with the addition of bicalutamide resulted in a greater reduction in PSA expression as compared to bicalutamide treatment alone (79%, lane 3 vs. lane 1). BRG1 depletion with bicalutamide treatment initiated a 69% drop in PSA expression compared to vehicle treated vector (lane 4 vs. lane 1). Furthermore, the TMPRSS2 AR target gene elicited the same trend as compared to PSA upon BRM (44% reduction) and BRG1 (34% reduction) knockdown in response to bicalutamide (data not shown). Thus, these data indicate that SWI/SNF function is likely dispensable for the response to AR antagonists.

To validate this supposition, the impact of BAF57 was examined (Fig. 3B). Experiments were performed as above after introduction of dominant negative BAF57 (BAF57 Δ N), as attempts to establish significant ablation of BAF57 in AR

positive prostate cancer cells have proven unsuccessful (data not shown). BAF57 Δ N is proposed to act through its ability to retain SWI/SNF association, but is unable to carry out its appropriate function due to the lack of the N-terminus (proline-rich and high mobility group DNA binding domains) (6). As expected, vehicle treatment and vector transfection demonstrated robust PSA mRNA expression, whereas the addition of bicalutamide caused a complete loss in expression (lane 1 and lane 2, respectively). Furthermore, inhibition of BAF57 function upon transfection of BAF57 Δ N showed a 63% reduction in PSA expression upon vehicle treatment compared to vector transfection (lane 3 vs. lane 1). Upon treatment with bicalutamide, disruption of BAF57 function provided no change in the ability of bicalutamide to repress AR activity. Thus, similar to what was shown after knockdown of BRM or BRG1, BAF57 activity was not necessary for bicalutamide-mediated repression of AR activity. Additionally, the requirement of BAF57 for bicalutamide-mediated suppression of AR dependent prostate cancer proliferation was examined (Fig. 3C). Upon transfection with control vector, BrdU incorporation analyses revealed a 42% reduction in LNCaP prostate cancer cell proliferation in response to treatment with bicalutamide compared to vehicle treatment. Consistent with previous reports (29), the cell cycle inhibitor p16^{ink4a} was utilized as the positive control, and demonstrated significant attenuation (72% for vehicle treated; 83% for bicalutamide treated) of LNCaP cell proliferation compared to vector control. Inhibition of BAF57 function demonstrated a 17% reduction in LNCaP cell proliferation upon vehicle treatment. Furthermore, bicalutamide treatment remained equally as effective at

inhibiting prostate cancer cell proliferation upon BAF57 Δ N transfection as compared to the vector transfected cells with the same treatment. Together, these data indicate that disruption of SWI/SNF or BAF57 alone does not compromise the response to AR antagonist. Given the established requirement of BAF57 for the response to AR agonists, these observations suggest that the SWI/SNF-AR interaction could be developed as a therapeutic target. However, the mechanisms underlying this association must first be determined.

BAF57 binds to the DNA binding domain and hinge region of AR.

Although it is clear that BAF57 can associate directly with AR, the mechanism of receptor association has not been identified. This is of importance, as current strategies to suppress AR function act through the receptor ligand binding domain. To identify the BAF57 association site, *in vitro* binding assays were performed (Fig. 4A). Consistent with previous reports (31), recombinant BAF57 associated with full length AR *in vitro* (top panel, compare lanes 2 and 3), but did not associate with CD44 (negative control). As shown, BAF57 showed no binding capacity to the N-terminus of AR (amino acids 1-502) whereas strong association with the C-terminus of AR (amino acids 506-919) was observed. The C-terminal domain of AR consists of several regions as depicted in Fig. 4A, top panel: the highly conserved (among nuclear receptors) DNA binding domain (DBD), the hinge region, and the ligand binding domain. To further characterize the region(s) of binding of BAF57 on AR, the DBD and hinge region within AR were explored, as these regions within the estrogen receptor (ER) have been implicated in BAF57 binding (16). As seen in Fig. 4B, BAF57 failed to bind to the first 173

amino acids of AR compared to GST control (lane 3 vs. 2). However, significant binding was observed between BAF57 and the DBD/hinge region (lane 4). Subsequent dissection showed that both the DBD and hinge region harbor some BAF57 binding capacity (lane 5 and 6), signifying that these combined motifs provide a means for BAF57 to exert its function on AR. Together, these data identify the AR DBD/hinge region as the principal site of BAF57 association and indicate that this interaction utilizes AR motifs not currently targeted in therapy.

Loss of CTE within AR causes reduction in BAF57 binding and transactivation potential. The observation that BAF57 binds the hinge domain was of importance as this region contains a C-terminal extension (CTE) which is necessary for AR binding to AREs (19, 42). To characterize the impact of the CTE on BAF57 mediated AR activation, a small, eight amino acid deletion within the CTE (amino acids 629-636) of AR was utilized in GST pull-down analyses. As shown in Fig. 5A, a 50% reduction was seen in BAF57 binding to AR Δ 629-636 as compared to wtAR, indicating that this region is of importance for complete BAF57 binding.

Following this finding, the functional importance of the AR CTE for BAF57 mediated activation of AR was examined through reporter analyses (Fig. 5B). BT549 cells (BAF57 null) were transfected as indicated and activation of wtAR or AR Δ 629-636 was measured through activity of an AR target promoter (probasin, ARR2) fused to luciferase. As expected, minimal activation of wtAR was detected in the absence of BAF57. Identical results were observed for AR Δ 629-636. Addition of BAF57 strongly induced activation of wtAR (13-fold), but not AR Δ 629-

636 (5-fold), despite equal protein expression (bottom panel). These data indicate that the CTE is critical for BAF57 mediated activation of AR, and that disrupting the BAF57-AR interaction may provide a means of attenuating AR activity.

Loss of BAF57 N-terminus disrupts binding to AR *in vitro*. Having determined the DBD/hinge and CTE regions of AR as primary interaction sites for BAF57, the interaction surface of AR within the domain(s) of BAF57 was elucidated. BAF57 is thought to serve as an architectural protein which mediates its action through several functional domains. As depicted in Fig. 6A, BAF57 consists of an N-terminal proline-rich (PR) region, a highly conserved high mobility group (HMG) DNA binding domain, and a kinesin-like coiled-coil domain. GST pull-down analysis was carried out with GST or the indicated GST-AR fusion proteins (Fig. 6B). The purified proteins were incubated with *in vitro* transcribed/translated BAF57 (full length) or BAF57 Δ N (lacking the PR and HMG domains). As expected, BAF57 exhibited minimal or no binding to GST (lane 2) and GST-AR 1-173 (lane 3). Additionally, significant binding was observed with GST-AR 1-660 (lane 4) and GST-AR 505-676 (lane 5), as both contain the DBD/hinge region shown to strongly bind BAF57 (Fig. 4A, B). To determine the impact of the N-terminus of BAF57 on AR binding, pull-down analysis was also carried out between BAF57 Δ N and the above-mentioned GST or GST-AR fusion proteins. As shown, no binding was apparent between BAF57 Δ N and any of the AR fusion proteins (lanes 2-5). This finding suggests that the N-terminal domain

of BAF57, containing the PR and the HMG domain, is the primary AR interaction site.

To further refine this mapping, subsequent *in vitro* pull-down analyses were carried out with GST-AR 505-676 and two additional BAF57 deletion mutants, BAF57 Δ PR and BAF57 Δ HMG (Fig. 6C). Interestingly, in the absence of the HMG domain, no effect on AR binding was observed (bottom panel and quantification at right, 98% of full-length BAF57). However, removal of the extreme N-terminal PR domain demonstrated an apparent 56% reduction in AR binding compared to full-length BAF57 binding (set at 100%; as determined from the combination of two independent experiments, right panel). As binding was not completely eliminated in the absence of the PR region, it is possible that a weak binding site remains within the HMG domain of BAF57. Thus, these data indicate that the primary site of AR interaction occurs within the N-terminus of BAF57, predominantly within the PR region.

N-terminal domain of BAF57 blocks AR activity and LNCaP cancer cell proliferation. Given the importance of the BAF57 N-terminus for direct AR association, the sufficiency of this region to bind AR was examined (Fig. 7A). As expected, BAF57 Δ N exhibited minimal binding to AR (bottom panel, 1.7% of input) compared to GST alone (lane 3 vs. lane 2). However, the BAF57 1-145 fragment supported AR association (top panel, 11.0% of input) compared to GST alone (lane 3 vs. lane 2). Thus, amino acids 1-145 are sufficient for AR association. Given this function, the ability of ectopic expression of BAF57 1-145 to disrupt BAF57-mediated AR activation was assessed. First, however,

expression of BAF57 1-145 3Xflag was validated using the Flag antibody, as the developed BAF57 antibody will not recognize this N-terminal peptide. Transfection of BAF57 1-145 3Xflag demonstrated effective expression and peptide accumulation in prostate cancer cells, as AR and levels of endogenous, full-length BAF57 remained unchanged (Fig. 7B, top panels). This differs from previous observations wherein expression of BAF57 mutants demonstrated reduction in full-length endogenous BAF57 expression (5, 6).

To examine the consequence of the direct interaction between AR and the BAF57 N-terminus on AR function *in vivo*, the impact of BAF57 1-145 overexpression on AR recruitment to the PSA locus was determined (bottom panels). As previously shown by our lab and others, AR is strongly recruited to AREs within the PSA enhancer/promoter (Fig. 2B and (43)), as is the BAF57 protein (Fig. 2A and (31)), in response to androgen. As determined through ChIP analyses, AR was recruited to the ARE upon vector (pcDNA3) transfection (bottom panels, lane 2). Ectopic expression of BAF57 1-145, however, significantly reduced the recruitment of AR (26% of control, as determined by Q-PCR) and BAF57 (data not shown) to the PSA enhancer element (bottom panels) as compared to recruitment in the presence of vector control. Thus, expression of the BAF57 AR binding fragment is sufficient to disrupt AR/BAF57 residence at AREs.

Given this observed decrease in AR recruitment, the impact of BAF57 1-145 overexpression on concomitant AR activity was determined (Fig. 7C). Reporter analyses were carried out in BT549 cells (as in Fig. 5B), wherein

ectopic expression of AR and BAF57 exhibited robust activation of AR in the presence of DHT (set to 100%). Expression of AR and BAF57 1-145, by contrast, showed minimal activation (12%). Upon introduction of increasing concentrations of BAF57 1-145 along with wtBAF57 and AR, a significant decrease in AR activity was seen (37% and 27%, respective of increasing concentration of BAF57 1-145), providing functional evidence for the ability of BAF57 1-145 to disrupt AR signaling. To further elucidate the ability of BAF57 1-145 to suppress AR transcriptional activity, endogenous AR activity was monitored (Fig. 7D). For these studies, expression of two well characterized prostate specific AR target genes (PSA and TMPRSS2) was examined. As expected, RT-PCR analyses revealed strong mRNA expression of PSA and TMPRSS2 in the presence of androgen (vector transfected AR target gene/GAPDH set to 1). Ectopic expression of the dominant negative AR (Δ 46-408, positive control) reduced the expression of PSA and TMPRSS2 to 65% and 75%, respectively. Additionally, expression of BAF57 1-145 caused a substantial reduction in PSA (41%) and TMPRSS2 (57%) relative to control. Together, these data demonstrate that overexpression of BAF57 1-145 significantly attenuates AR transcriptional activity on ectopic and endogenous AR targets.

Given the requirement of AR transcriptional activity for the proliferation of prostate cancer cells, the effect of BAF57 1-145 expression on prostate cancer cell proliferation was tested (Fig. 7E). The LNCaP cell line was utilized and transfected with H2B-GFP (to mark transfection efficiency) and either vector control (pcDNA3), dnAR, or BAF57 1-145. As expected, dnAR demonstrated

significant inhibition of LNCaP cell proliferation (26%) over vector control. Similarly, addition of BAF57 1-145 was able to significantly attenuate LNCaP prostate cancer cell growth (31%) as compared to vector control. Together, these data demonstrate the ability of BAF57 1-145 to disrupt AR recruitment, transcriptional activation, and prostate cancer cell proliferation, providing the groundwork for the development of a potential therapeutic target for the disruption of the BAF57-AR interaction and consequently prostate cancer cell proliferation.

Discussion

Given the importance of BAF57 for AR signaling, the present study was designed to delineate the mechanisms by which BAF57 governs AR specificity of function, and to exploit the significance of this event for AR-dependent prostate cancer proliferation. Screening of primary and metastatic tumors revealed that BAF57 expression is retained and often elevated in this disease (Fig. 1); these data are in agreement with the known requirement of prostate cancers for sustained AR signaling, and the proposed influence of BAF57 on this event. Kinetic studies demonstrated that BAF57 is indeed recruited early to AREs after ligand stimulation, thus indicating that BAF57 likely acts early to assist in initiating transcriptional activation (Fig. 2). However, while BAF57 is necessary for the response to agonist, BAF57 (or SWI/SNF) function proved dispensable for the response to AR antagonists used in therapy (Fig. 3). Together, these data suggest that BAF57 may serve as a useful biochemical target to inhibit AR function in tumor cells. To move toward this goal, mechanistic studies revealed that BAF57 binds to a critical region of AR that is known to be required for agonist function, the DBD/hinge region (Fig. 4, 5); importantly, this motif of AR is not currently targeted in therapy. The ability to bind AR was shown to be mediated by the proline rich/ HMG domains of BAF57 (Fig. 6). Furthermore, expression of the AR binding motif in cells was sufficient to reduce AR residence at endogenous targets, suppress AR-dependent gene activation, and inhibit prostate cancer cell proliferation (Fig. 7). Together, these findings reveal the means by which BAF57 regulates AR activity, and identify the AR-BAF57

association as an effective target for the development of means to suppress AR function.

It is well understood that the SWI/SNF chromatin remodeling complex is involved in the activation of gene transcription as well as repression of certain target genes. The differential requirement of SWI/SNF for the response to AR agonists versus antagonists is distinct from that observed with other nuclear receptors. For example, the BRG1/BRM ATPases are necessary for antagonist mediated repression of the estrogen receptor (ER) (53, 54). This response was shown to be dependent upon function of the putative tumor suppressor, prohibitin, through E2F suppression. By contrast, our data show that bicalutamide exhibited substantial repression of AR activity despite loss of either SWI/SNF ATPase or inhibition of BAF57. These findings are in strong accordance with recent evidence demonstrating that BRM is not recruited to AREs in response to AR antagonists (28). Additionally, the N-CoR co-repressor complex has already emerged as a critical means of eliciting bicalutamide repression of AR, and although BRG1 recruitment to the PSA promoter was observed with HDAC3 upon bicalutamide stimulation (57), we were unable to determine any functional role of this ATPase for antagonist mediated repression. Furthermore, the observation that SWI/SNF is dispensable for antagonist mediated repression of AR is supported by the ability of bicalutamide to disrupt NH₂/COOH terminal interactions within AR (22, 33), as this has not been shown to require SWI/SNF activity. The above findings reveal a selective response by the SWI/SNF complex in regulating AR agonist mediated function, with a

dispensable requirement for repression in response to antagonist. This non-redundant nature of SWI/SNF underscores the potential to identify the key components utilized by the complex to perform selective signaling processes and may allow for the targeted development of certain components involved in the progression of specific diseases such as prostate cancer.

In accordance with these ideas, the observed early recruitment of BAF57 to the sites of AR activity and its subsequent displacement indicates that SWI/SNF may be necessary to facilitate initial events in transcriptional activation. This supposition is in agreement with the proposed mechanisms of active SWI/SNF remodeling function, in that the complex is thought to progress along the nucleosome bound DNA (away from the AREs) disrupting this contact by either nucleosomal sliding along the DNA or transfer of the histone octamers to another region of DNA (14, 35). This action is carried out through the central ATPase domain of BRM or BRG1, generating an open chromatin structure and thereby permitting the binding of basal transcriptional machinery and the progressive action of pol II (21, 35, 47). Of note is that intermittent signaling of SWI/SNF has also been demonstrated for transcriptional activation of GR responsive genes, wherein GR serves to recruit BRG1 to permit activation, which is subsequently followed by displacement of the receptor by SWI/SNF (15, 34). Similarly, AR is able to induce recruitment of SWI/SNF (28), presumably through the BAF57 interaction motif as shown herein; however, in contrast to SWI/SNF displacement of GR at specific GREs, AR residence is stable at the PSA locus. These data indicate that nuclear receptor displacement is not requisite for

SWI/SNF dismissal. It has been proposed that retention of AR at the PSA enhancer permits looping and contact with the proximal promoter, thus augmenting transcriptional output (52). The observation that dismissal of BAF57 occurs indicates that SWI/SNF may no longer be required after this looping event occurs, and future investigations will test this hypothesis directly. Given the dynamic interplay between AR and BAF57, it was essential to delineate the biochemical mechanisms that underpin BAF57 recruitment.

As has been well-documented, AR contains a zinc finger DNA binding domain that requires a C-terminal extension (CTE) within the hinge domain to permit binding to DNA (18, 19, 42). A closer examination of the AR CTE uncovered this region as an important region for BAF57 binding and activation of AR (Fig. 5). Interestingly, there is precedence for a requirement of the CTE to confer the binding of HMG DBD containing proteins (HMGB1 and SOX9) to AR (50, 51). SOX9, specifically, is overexpressed in recurrent prostate cancer and regulates AR expression levels. Moreover, the DBD/hinge domain within the estrogen receptor (ER) has also been shown to be a primary region for BAF57 interaction, indicating a possible trend in BAF57-nuclear receptor binding (16). Since our data demonstrate that the AR binding region of BAF57 (amino acids 1-145) is able to reduce AR residence at AREs, it is possible that binding of the BAF57 fragment to the CTE is sufficient to block either DNA binding or agonist induced stabilization on DNA. While additional investigations will be necessary to challenge this hypothesis, the current data strongly support ablation of the AR-BAF57 interaction as a means to disrupt AR-DNA association.

To move further toward the goal of inhibiting the AR-BAF57 interaction, the region of AR binding within BAF57 was defined. As described, biochemical analyses revealed the PR and HMG domains of BAF57 as the primary binding sites for AR, as deletion of these regions elicited a loss in AR binding. Interestingly, the observed binding to AR differs from that of ER association with BAF57, wherein truncation of either the N- or C-terminus of BAF57 caused significant disruption of ER binding, indicating that the intact protein is required for interaction (16). Thus, the AR and ER have quite distinct interaction schemes which further reveal the selective specificity of the SWI/SNF complex and may lead to potential functional differences in response to BAF57. Additionally, these BAF57 domains necessary for AR binding are dispensable for association with the CoREST neuronal gene co-repressor. Rather, association with CoREST, which is required for REST/CoREST mediated gene repression, is driven by the kinesin-like coiled-coil motif (3). Similarly, the PR/HMG domain of BAF57 is also dispensable for association with BRG1/BRM (6) and the BAF155 subunit of SWI/SNF, as this interaction was also dependent upon the coiled-coil domain as well as the conserved NHRLI motif of BAF57 located in the central region of the protein (5). This latter observation may have implications for AR, as BAF155 (and its murine homolog, SRG3) has been shown to regulate expression of specific SWI/SNF components, including BAF57, and is thought to serve as a critical regulator of SWI/SNF subunit levels (5, 44). As would be expected, therefore, SRG3 has been shown to be important for AR regulation (23).

Given the necessary role for the BAF57 N-terminus in AR binding and the ability of this region to disrupt AR recruitment, utility of the BAF57 N-terminus as a means to suppress AR activity was examined. As shown, AR activity was significantly attenuated and proliferation of the prostate cancer cells was diminished. Importantly, introduction of the BAF57 N-terminal region caused no reduction of endogenous full-length BAF57 protein expression. This is in contrast to previous observations that demonstrated a decrease in wtBAF57 expression upon BAF57 mutant introduction (5, 6). Therefore, effects observed upon addition of this AR binding region are not a result of decreased wtBAF57 expression. Taken together, these data present the BAF57-AR interface as a medium for disrupting AR signaling. These findings are of significance as recent research has validated the feasibility of developing small molecules to effectively disrupt protein-protein interaction in cancer. For example, the Nutlin compounds were developed to block MDM2 interaction with p53, consequently stabilizing the p53 tumor suppressor and inducing cell cycle arrest and apoptosis (49). Furthermore, expression of the N-terminal region of AR (amino acids 1-558) has been demonstrated to block the growth of prostate cancer through unknown mechanisms (38). These studies provide much impetus to explore disruption of the AR-BAF57 interaction through small molecule inhibitor and/or peptide exposure to thwart prostate cancer proliferation.

In summary, the robust expression of BAF57 in a multitude of prostate cancer specimens and its dependence on the SWI/SNF complex strongly suggest that the integrity of BAF57 function is maintained in prostate cancer as a

means to support AR activity. Specificity of action was revealed, in that BAF57 appears selectively required for the response to agonist (but not antagonist), and acts rapidly at AR target sites upon receptor activation. Disruption of this event, made possible through dissection of the AR-BAF57 interaction surfaces, proved sufficient to disrupt AR residence on chromatin, AR-dependent gene expression, and prostate cancer cell proliferation. Together, these data demonstrate that disruption of the BAF57-AR interaction may prove an effective, novel means to suppress AR function and cellular outcome in prostate cancer cells.

Acknowledgments

This work was supported by R01CA116777-02 and R21CA121402-02 (K. E. K.). K. A. L. is supported by Department of Defense grant W81XWH-06-1-0098.

The authors thank Dr. Erik Knudsen, Dr. Lisa Morey, Matthew Schiewer, and Ankur Sharma for critical reading of the manuscript, Dr. Sohaib Khan, Dr. Jinsong Zhang, Dr. Carolyn Price, and Dr. Bernard Weissman for critical discussions, and Dr. Bernard Weissman for the BRG1i and BRMi expression vectors, and Dr. Trevor Archer for the BAF57 Δ PR and BAF57 Δ HMG expression vectors.

References

1. **Allred, D. C., G. M. Clark, R. Elledge, S. A. Fuqua, R. W. Brown, G. C. Chamness, C. K. Osborne, and W. L. McGuire.** 1993. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst* **85**:200-6.
2. **Balk, S. P.** 2002. Androgen receptor as a target in androgen-independent prostate cancer. *Urology* **60**:132-8; discussion 138-9.
3. **Battaglioli, E., M. E. Andres, D. W. Rose, J. G. Chenoweth, M. G. Rosenfeld, M. E. Anderson, and G. Mandel.** 2002. REST repression of neuronal genes requires components of the hSWI.SNF complex. *J Biol Chem* **277**:41038-45.
4. **Brinkmann, A. O., L. J. Blok, P. E. de Ruiter, P. Doesburg, K. Steketee, C. A. Berrevoets, and J. Trapman.** 1999. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* **69**:307-13.
5. **Chen, J., and T. K. Archer.** 2005. Regulating SWI/SNF subunit levels via protein-protein interactions and proteasomal degradation: BAF155 and BAF170 limit expression of BAF57. *Mol Cell Biol* **25**:9016-27.
6. **Chi, T. H., M. Wan, K. Zhao, I. Taniuchi, L. Chen, D. R. Littman, and G. R. Crabtree.** 2002. Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature* **418**:195-9.

7. **Comstock, C. E., and K. E. Knudsen.** 2007. The complex role of AR signaling after cytotoxic insult: implications for cell-cycle-based chemotherapeutics. *Cell Cycle* **6**:1307-13.
8. **Decristofaro, M. F., B. L. Betz, C. J. Rorie, D. N. Reisman, W. Wang, and B. E. Weissman.** 2001. Characterization of SWI/SNF protein expression in human breast cancer cell lines and other malignancies. *J Cell Physiol* **186**:136-45.
9. **Dehm, S. M., and D. J. Tindall.** 2007. Androgen Receptor Structural and Functional Elements: Role and Regulation in Prostate Cancer. *Mol Endocrinol*.
10. **Edwards, M., J. Twin, and S. Wilkinson.** 2002. New technique to assess the axilla for breast cancer metastases using cell separation technology. *ANZ J Surg* **72**:655-9.
11. **Epstein, J. I., W. C. Allsbrook, Jr., M. B. Amin, and L. L. Egevad.** 2006. Update on the Gleason grading system for prostate cancer: results of an international consensus conference of urologic pathologists. *Adv Anat Pathol* **13**:57-9.
12. **Feldman, B. J., and D. Feldman.** 2001. The development of androgen-independent prostate cancer. *Nat Rev Cancer* **1**:34-45.
13. **Flajollet, S., B. Lefebvre, C. Cudejko, B. Staels, and P. Lefebvre.** 2007. The core component of the mammalian SWI/SNF complex SMARCD3/BAF60c is a coactivator for the nuclear retinoic acid receptor. *Mol Cell Endocrinol* **270**:23-32.

14. **Flaus, A., and T. Owen-Hughes.** 2001. Mechanisms for ATP-dependent chromatin remodelling. *Curr Opin Genet Dev* **11**:148-54.
15. **Fletcher, T. M., N. Xiao, G. Mautino, C. T. Baumann, R. Wolford, B. S. Warren, and G. L. Hager.** 2002. ATP-dependent mobilization of the glucocorticoid receptor during chromatin remodeling. *Mol Cell Biol* **22**:3255-63.
16. **Garcia-Pedrero, J. M., E. Kiskinis, M. G. Parker, and B. Belandia.** 2006. The SWI/SNF chromatin remodeling subunit BAF57 is a critical regulator of estrogen receptor function in breast cancer cells. *J Biol Chem* **281**:22656-64.
17. **Gelmann, E. P.** 2002. Molecular biology of the androgen receptor. *J Clin Oncol* **20**:3001-15.
18. **Haelens, A., T. Tanner, S. Denayer, L. Callewaert, and F. Claessens.** 2007. The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor. *Cancer Res* **67**:4514-23.
19. **Haelens, A., G. Verrijdt, L. Callewaert, V. Christiaens, K. Schauwaers, B. Peeters, W. Rombauts, and F. Claessens.** 2003. DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation. *Biochem J* **369**:141-51.
20. **He, B., J. A. Kemppainen, and E. M. Wilson.** 2000. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. *J Biol Chem* **275**:22986-94.

21. **Heinlein, C. A., and C. Chang.** 2002. Androgen receptor (AR) coregulators: an overview. *Endocr Rev* **23**:175-200.
22. **Hodgson, M. C., I. Astapova, A. N. Hollenberg, and S. P. Balk.** 2007. Activity of Androgen Receptor Antagonist Bicalutamide in Prostate Cancer Cells Is Independent of NCoR and SMRT Corepressors. *Cancer Res* **67**:8388-95.
23. **Hong, C. Y., J. H. Suh, K. Kim, E. Y. Gong, S. H. Jeon, M. Ko, R. H. Seong, H. B. Kwon, and K. Lee.** 2005. Modulation of androgen receptor transactivation by the SWI3-related gene product (SRG3) in multiple ways. *Mol Cell Biol* **25**:4841-52.
24. **Hsiao, P. W., C. J. Fryer, K. W. Trotter, W. Wang, and T. K. Archer.** 2003. BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. *Mol Cell Biol* **23**:6210-20.
25. **Huang, Z. Q., J. Li, L. M. Sachs, P. A. Cole, and J. Wong.** 2003. A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. *Embo J* **22**:2146-55.
26. **Inoue, H., T. Furukawa, S. Giannakopoulos, S. Zhou, D. S. King, and N. Tanese.** 2002. Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. *J Biol Chem* **277**:41674-85.
27. **Jenster, G.** 1999. The role of the androgen receptor in the development and progression of prostate cancer. *Semin Oncol* **26**:407-21.

28. **Klokk, T. I., P. Kurys, C. Elbi, A. K. Nagaich, A. Hendarwanto, T. Slagsvold, C. Y. Chang, G. L. Hager, and F. Saatcioglu.** 2007. Ligand-specific dynamics of the androgen receptor at its response element in living cells. *Mol Cell Biol* **27**:1823-43.
29. **Knudsen, K. E., K. C. Arden, and W. K. Cavenee.** 1998. Multiple G1 regulatory elements control the androgen-dependent proliferation of prostatic carcinoma cells. *J Biol Chem* **273**:20213-22.
30. **Lapointe, J., C. Li, J. P. Higgins, M. van de Rijn, E. Bair, K. Montgomery, M. Ferrari, L. Egevad, W. Rayford, U. Bergerheim, P. Ekman, A. M. DeMarzo, R. Tibshirani, D. Botstein, P. O. Brown, J. D. Brooks, and J. R. Pollack.** 2004. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci U S A* **101**:811-6.
31. **Link, K. A., C. J. Burd, E. Williams, T. Marshall, G. Rosson, E. Henry, B. Weissman, and K. E. Knudsen.** 2005. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. *Mol Cell Biol* **25**:2200-15.
32. **Marshall, T. W., K. A. Link, C. E. Petre-Draviam, and K. E. Knudsen.** 2003. Differential requirement of SWI/SNF for androgen receptor activity. *J Biol Chem* **278**:30605-13.
33. **Masiello, D., S. Cheng, G. J. Bubley, M. L. Lu, and S. P. Balk.** 2002. Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. *J Biol Chem* **277**:26321-6.

34. **Nagaich, A. K., D. A. Walker, R. Wolford, and G. L. Hager.** 2004. Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodeling. *Mol Cell* **14**:163-74.
35. **Narlikar, G. J., H. Y. Fan, and R. E. Kingston.** 2002. Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**:475-87.
36. **Nie, Z., Y. Xue, D. Yang, S. Zhou, B. J. Deroo, T. K. Archer, and W. Wang.** 2000. A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. *Mol Cell Biol* **20**:8879-88.
37. **Phelan, M. L., S. Sif, G. J. Narlikar, and R. E. Kingston.** 1999. Reconstitution of a core chromatin remodeling complex from SWI/SNF subunits. *Mol Cell* **3**:247-53.
38. **Quayle, S. N., N. R. Mawji, J. Wang, and M. D. Sadar.** 2007. Androgen receptor decoy molecules block the growth of prostate cancer. *Proc Natl Acad Sci U S A* **104**:1331-6.
39. **Rhodes, D. R., J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, T. Barrette, A. Pandey, and A. M. Chinnaiyan.** 2004. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* **6**:1-6.
40. **Rosson, G. B., C. Bartlett, W. Reed, and B. E. Weissman.** 2005. BRG1 loss in MiaPaCa2 cells induces an altered cellular morphology and disruption in the organization of the actin cytoskeleton. *J Cell Physiol* **205**:286-94.

41. **Scher, H. I., and C. L. Sawyers.** 2005. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol* **23**:8253-61.
42. **Schoenmakers, E., P. Alen, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts, and F. Claessens.** 1999. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. *Biochem J* **341 (Pt 3)**:515-21.
43. **Shang, Y., M. Myers, and M. Brown.** 2002. Formation of the androgen receptor transcription complex. *Mol Cell* **9**:601-10.
44. **Sohn, D. H., K. Y. Lee, C. Lee, J. Oh, H. Chung, S. H. Jeon, and R. H. Seong.** 2007. SRG3 interacts directly with the major components of the SWI/SNF chromatin remodeling complex and protects them from proteasomal degradation. *J Biol Chem* **282**:10614-24.
45. **Sun, Z., J. Pan, and S. P. Balk.** 1997. Androgen receptor-associated protein complex binds upstream of the androgen-responsive elements in the promoters of human prostate-specific antigen and kallikrein 2 genes. *Nucleic Acids Res* **25**:3318-25.
46. **Taplin, M. E.** 2007. Drug insight: role of the androgen receptor in the development and progression of prostate cancer. *Nat Clin Pract Oncol* **4**:236-44.

47. **Urnov, F. D., and A. P. Wolffe.** 2001. Chromatin remodeling and transcriptional activation: the cast (in order of appearance). *Oncogene* **20**:2991-3006.
48. **Vanaja, D. K., J. C. Cheville, S. J. Iturria, and C. Y. Young.** 2003. Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res* **63**:3877-82.
49. **Vassilev, L. T., B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, and E. A. Liu.** 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**:844-8.
50. **Verrijdt, G., A. Haelens, E. Schoenmakers, W. Rombauts, and F. Claessens.** 2002. Comparative analysis of the influence of the high-mobility group box 1 protein on DNA binding and transcriptional activation by the androgen, glucocorticoid, progesterone and mineralocorticoid receptors. *Biochem J* **361**:97-103.
51. **Wang, H., N. C. McKnight, T. Zhang, M. L. Lu, S. P. Balk, and X. Yuan.** 2007. SOX9 is expressed in normal prostate basal cells and regulates androgen receptor expression in prostate cancer cells. *Cancer Res* **67**:528-36.
52. **Wang, Q., J. S. Carroll, and M. Brown.** 2005. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. *Mol Cell* **19**:631-42.

53. **Wang, S., B. Zhang, and D. V. Faller.** 2004. BRG1/BRM and prohibitin are required for growth suppression by estrogen antagonists. *Embo J* **23**:2293-303.
54. **Wang, S., B. Zhang, and D. V. Faller.** 2002. Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. *Embo J* **21**:3019-28.
55. **Wetherill, Y. B., C. E. Petre, K. R. Monk, A. Puga, and K. E. Knudsen.** 2002. The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells. *Mol Cancer Ther* **1**:515-24.
56. **Yuan, X., M. L. Lu, T. Li, and S. P. Balk.** 2001. SRY interacts with and negatively regulates androgen receptor transcriptional activity. *J Biol Chem* **276**:46647-54.
57. **Zhu, P., S. H. Baek, E. M. Bourk, K. A. Ohgi, I. Garcia-Bassets, H. Sanjo, S. Akira, P. F. Kotel, C. K. Glass, M. G. Rosenfeld, and D. W. Rose.** 2006. Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell* **124**:615-29.

Figure Legends

FIG. 1. BAF57 is highly expressed in human prostate cancer specimens.

(A) Immunoblotting was performed for BAF57 and lamin B (loading control) in the LNCaP, LAPC4, 22RV1, DU-145, and PC-3 prostate cancer cells. BT549 cells were used as the negative control. (B) Specificity of the BAF57 antibody was determined by IHC analysis utilizing tissue sections from paraffin embedded BT549 and LNCaP cell pellets. Antibody was used at a dilution of 1:2000. (C) Non-neoplastic (top and middle panels) and primary human prostate cancer (bottom panel) was analyzed for BAF57 expression by IHC. No primary, top panel. BAF57 staining (1:2000), middle and bottom panels. (D) Two lymph node (LN) metastases of prostate cancer were stained by standard H & E (left panels), AR (middle panels), or BAF57 (right panels). (E) IHC staining was performed for BAF57 on a tissue microarray slide containing approximately 80 cores. “n” number for each category is indicated. BAF57 IHC score is displayed as a composite of intensity + extent of tumor staining as indicated in Materials and Methods section.

FIG. 2. BAF57 is intermittently recruited to androgen responsive elements.

LB1-Luc cells were cultured in steroid-free medium for 3 days and then treated with either 0.1% EtOH or 10nM DHT for the indicated time. Cells were harvested for ChIP analyses and antibodies to AR (A), BAF57 (B), or preimmune serum (IgG) were utilized to examine recruitment to the PSA enhancer region. At least

three independent experiments were performed and a representation is depicted. Band quantification for BAF57 recruitment is shown (B, bottom panel).

FIG. 3. SWI/SNF ATPases and BAF57 are not required for bicalutamide mediated repression of AR activity. (A, left panels) LNCaP cells were transfected with limiting H2B-GFP and pHTP-scrambled control, pHTP-BRMi, or pHTP-BRG1i. Following transfection, cells were harvested and subjected to immunoblot analyses for BRM, BRG1, or CDK4 (control). Band quantification is indicated. (A, right panels) LNCaP prostate cancer cells were transfected with limiting H2B-GFP and pHTP-vector, pHTP-BRMi, or pHTP-BRG1i. Cells were treated with 0.1% EtOH or 1 μ M bicalutamide as indicated for 48h. Cells were harvested and RNA was isolated, and then subjected to RT-PCR. cDNA was amplified for PSA or GAPDH and band quantification of PSA over GAPDH is shown. Representative data from at least three experiments is shown. (B) LNCaP prostate cancer cells were transfected with H2B-GFP and pcDNA3-vector or pBabe-BAF57 Δ N-flag expression plasmid. Cells were treated with either 0.1% EtOH or 1 μ M bicalutamide as indicated for 48h (with re-stimulation after 24h). Cells were harvested and RNA was isolated, and then subjected to RT-PCR. cDNA was amplified for PSA or GAPDH and plotted as PSA over GAPDH (lower panel). Representative data from at least two independent experiments is shown. (C) LNCaP cells were transfected with H2B-GFP and pcDNA3-vector, pBabe-BAF57 Δ N-flag, or pCMV-p16ink4a. Following transfection, cells were treated with 0.1% EtOH or 1 μ M bicalutamide as indicated

for 48h (with re-stimulation after 24h). Bromodeoxyuridine (BrdU) was added for a 16h pulse, cells were fixed, and staining for BrdU incorporation was performed. Results from at least two independent experiments are plotted as percent BrdU incorporation relative to vector plus vehicle. * = $p < 0.05$.

FIG. 4. BAF57 binds AR DBD/hinge region. (A, top panel) Schematic representation of androgen receptor functional domains. (Bottom panel) Immobilized GST or GST-BAF57 was incubated with [³⁵S]-Met-labeled AR mutants or CD44 (negative control) overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Representative Coomassie of purified GST or GST-BAF57 is shown. (B, top panel) Schematic representation of GST-AR fusion proteins utilized in GST pull-down analyses. (Bottom panel) Immobilized GST or GST-AR fusions were incubated with [³⁵S]-Met-labeled BAF57 overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Coomassie of purified GST or GST-AR deletions is shown.

FIG. 5. Loss of C-terminal extension within AR hinge diminishes binding to BAF57 and transactivation potential. (A) Immobilized GST or GST-BAF57 was incubated with [³⁵S]-Met-labeled wtAR or CTE mutant of AR (AR Δ 629-636) overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Representative Coomassie of purified GST or GST-BAF57 is shown. Binding as a percentage to wtAR is shown

(bottom panel). (B) BT549 cells were transfected with 0.5 μ g pARR2-Luc and 0.25 μ g pTK-Renilla luciferase (for normalization) and 0.5 μ g pSG5AR, 0.5 μ g pSG5AR Δ 629-636, or 2.25 μ g pBabe-BAF57-flag as indicated. Post transfection, cells were treated with either 0.1% EtOH or 0.1nM DHT for 24h. Cells were harvested, lysed, and luciferase activity was measured using the Dual Luciferase Reporter Assay System. DHT stimulated wtAR activity is set to “1” and data are presented as relative luciferase activity. * = $p < 0.05$. (Bottom panel) BT549 cells were transfected with 0.5 μ g H2B-GFP and 1.5 μ g of pcDNA3 vector, pSG5AR, or pSG5AR Δ 629-636. Immunoblot analysis was performed for AR and CDK4.

FIG. 6. BAF57 N-terminus is sufficient for AR association. (A) Schematic representation of BAF57 domains and deletion constructs used. (B) Immobilized GST or GST-AR fusions were incubated with [35 S]-Met-labeled BAF57 or BAF57 Δ N overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Representative Coomassie of purified GST or GST-AR deletions is shown. (C) Immobilized GST or GST-AR fusions were incubated with [35 S]-Met-labeled BAF57, BAF57 Δ N, BAF57 Δ PR, or BAF57 Δ HMG overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Binding as a percentage to wtBAF57 is shown (right panel). (D) HEK293 cells were transfected with H2B-GFP, pSG5AR and pcDNA3 vector, pcDNA3-BAF57-flag, pcDNA3-BAF57 Δ N-flag, pcDNA3-BAF57 Δ PR-flag, or pcDNA3-BAF57 Δ HMG-flag. Post transfection, cells were harvested, lysed, and coimmunoprecipitation analyses were

performed with M2-flag resin and equal concentrations of lysate. Input and bound samples were subjected to SDS-PAGE and AR was detected through immunoblot analysis.

FIG. 7. BAF57 N-terminus reduces AR residence at AREs, limits AR activity, and suppresses prostate cancer cell proliferation. (A, left panel) Schematic representation of BAF57, BAF57 1-145, and BAF57 Δ N is shown. Immobilized GST or GST-AR 505-676 were incubated with [³⁵S]-Met-labeled BAF57 1-145 or BAF57 Δ N overnight at 4°C, washed, and subjected to SDS-PAGE. 5% input and bound protein was detected by autoradiography. Binding as a percent of input is shown at right. (B) LNCaP cells were transfected with 2 μ g H2B-GFP and 8 μ g of either pcDNA3 or pcDNA3-BAF57 1-145 3Xflag. Post transfection, medium was changed to steroid free conditions for 48h. Cells were stimulated with 10nM DHT for 1h and subjected to immunoblot analysis for Flag, BAF57, or AR (top panel) or ChIP analysis for AR recruitment to the PSA enhancer region (bottom panels). (Bottom panels) 10% input and AR recruitment is shown. (C) BT549 cells (1x10⁵ cells in 12 well dishes) were transfected with 0.25 μ g pARR2-Luc, 0.125 μ g pTK-Renilla luciferase (for normalization), 0.25 μ g pSG5AR, 1.125 μ g pBabe-BAF57-flag, and pcDNA3-BAF57 1-145 (at 0.5 μ g, 1.125 μ g, or 2.25 μ g) as indicated. Post transfection, cells were treated with either 0.1% EtOH or 0.1nM DHT for 24h. Cells were harvested, lysed, and luciferase activity was measured using the Dual Luciferase Reporter Assay System. DHT stimulated AR plus BAF57 is set to 100 and data are presented as relative luciferase activity. * = p < 0.05. (D) LNCaP

cells were transfected with H2B-GFP and plasmid encoding pcDNA3 vector, pSG5AR Δ 46-408 (dnAR), or pcDNA3-BAF57 1-145 3Xflag. Cells were harvested and RNA was isolated, and then subjected to RT-PCR. cDNA was amplified for PSA, TMPRSS2, or GAPDH and percent PSA or TMPRSS2 expression (band quantification) relative to GAPDH with vector control is set to 1. Representative data from at least three independent experiments is shown. (E) LNCaP cells were transfected with H2B-GFP and pcDNA3, pSG5AR Δ 46-408 (dnAR), or pcDNA3-BAF57 1-145 3Xflag. 48h post transfection, bromodeoxyuridine (BrdU) was added for a 16h pulse, cells were fixed, and staining for BrdU incorporation was performed. Results from at least three independent experiments are plotted as percent inhibition of BrdU incorporation over vector control. ** = $p < 0.01$.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Supplemental Figure 1

BAF57 expression is increased in metastatic prostate cancer specimens. Differential expression profiling of BAF57 was examined utilizing two independent prostate tissue datasets containing the indicated number of normal, cancer, and metastatic samples. Datasets were obtained from the Oncomine microarray database and were selected based on having adequate “n” numbers for normal, cancer, and metastatic samples for comparison. Rhodes D. R. et. al. Oncomine: a cancer microarray database and integrated data-mining platform. Neoplasia 6, 1-6. (2004).

Chapter V

Summary and Future Directions:

Androgen Receptor Signaling as a Therapeutic Target

The androgen receptor (AR) and its associated activity is typically restored and/or elevated in recurrent prostate cancer (1, 2). Thus, much of the current research surrounding prostate cancer encompasses examining the AR signaling pathway and the necessary involvement of additional factors that aid in transcriptional activation of the receptor. Identification of novel therapeutic targets is the primary goal of this type of research, which would allow for additional, more effective options for the treatment of recurrent disease. The data herein have examined one essential aspect of the AR activation pathway. Based on preliminary results in the lab and elsewhere, wherein the BRM central ATPase of the SWI/SNF chromatin remodeling complex was shown to be preferentially required for AR activation (3, 4), the impact of the BAF57 SWI/SNF subunit was challenged as a critical regulator of the AR activation pathway.

SWI/SNF is Critical for AR Activity

The BRM ATPase is preferentially required for AR mediated activity and prostate cancer cell proliferation (Chapter II and others) (3-5). Contrary to this, BRG1 was demonstrated to suppress AR activation as shown through inhibition of the ATPase which lead to an increase in PSA expression (data not shown). Given the specificity of BRM for AR activation, it is enticing to speculate the possibility of targeting this ATPase in the treatment of prostate cancer. However, the underlying mechanism driving the specific action of either ATPase in regards

to AR transcriptional regulation has yet to be determined. Contributing to the evidence of ATPase specificity is that BRM expression is typically observed in differentiated cells such as prostate epithelia, in contrast to the BRG1 ATPase that is typically associated with undifferentiated tissue (6, 7). Additionally, BRG1 has been shown to associate with several zinc-finger DBD proteins. Given that AR contains a zinc-finger DBD, the possibility remains that BRG1 is able to directly elicit its putative repressive function on AR through binding of the AR DBD. Conversely, the inability of BRM to directly interact with AR (Chapter III) would suggest that it is targeted to AR (and specific target genes) via alternative means, such as select subunit association. Despite the promising *in vitro* models demonstrating the requirement of BRM on AR activation and prostate cancer proliferation, recent evidence has examined the expression of BRM in several human prostate cancer specimens demonstrating reduced overall expression, whereas BRG1 levels were increased (8). Data from our lab are consistent with these observations, demonstrating that BRM deficient mice develop areas of hyperplasia within the prostate (unpublished observations). Together, these data suggest that BRM may be acting as a potential tumor suppressor, wherein loss leads to uncontrolled proliferation. Alternatively, the reduced expression of BRM in human prostate cancer may be a result of the de-differentiation of the cancerous tissue, as BRM is primarily expressed in differentiated tissue as indicated above. However, the observations of BRM expression in human tumors do not rule out the potential for BRM to actually contribute to the initiation of prostate cancer prior to decreased expression levels, despite being unnecessary

for tumor maintenance. Regardless, the decrease in BRM expression in human prostate cancer would indicate that targeting the BRM ATPase directly may not prove to be an effective means of treating prostate cancer. Although the BRM ATPase has yet to have a clearly defined role in prostate cancer, the data presented suggest the involvement of additional SWI/SNF subunits in the regulation of AR activity and prostate cancer proliferation.

The BAF57 Subunit Mediates SWI/SNF Dependent AR Activity

Prior to the work presented here, the impact of BAF57 on AR activation was unknown. Initial studies established the ability of BAF57 to directly bind AR *in vitro* in the absence as well as the presence of ligand (Chapter III). Despite the observed ligand independent binding *in vitro*, the response this subunit has in a culture model system is clearly ligand dependent as indicated by recruitment of BAF57 to AREs in response to DHT. Recruitment of BAF57 leads to strong activation of AR target genes, a function of BAF57 that was shown to be dependent on BRM. In contrast to its ability to mediate AR activation, BAF57 has little influence on ER activity alone, primarily functioning through p160 coactivator mediated ER target gene transcription (9). This suggests a non-overlapping function for BAF57 and indicates possible specificity of action. Although it is clear that BAF57 is critical in AR target gene regulation and prostate cancer proliferation, the mechanism of action has remained elusive. Previous studies have described BAF57 as an “architectural” protein that consists of an N-terminal proline-rich (PR) and high mobility group (HMG) DNA binding domain (DBD), a kinesin-like coiled coil motif, and a C-terminal acidic region (10). As such, BAF57

has high affinity for four-way junction DNA (10). Thus, it is suspected that BAF57 plays a significant role in recruiting SWI/SNF to target DNA. BAF57 has been shown to interact with several transcriptional regulators, imposing differential regulation on several target genes. For instance, BAF57 assists in silencing CD4 expressing T-cells, but assists in activating CD8 expressers (11). As can be inferred, BAF57 exhibits gene specific responses, and although the specific mechanism is unclear, one possibility is through the ability of BAF57 to interact with an abundant number of proteins through differing domains, generating differential downstream responses. Clearly, much remains to be understood for determining the ability of BAF57 to exhibit targeted specificity.

As an additional means of promoting specificity, the HMG domain of BAF57 highly resembles the HMG1/2 proteins that have been reported to assist AR in DNA binding (12), indicating that BAF57 also may assist in AR stabilization and/or recruitment to DNA. Contrary to its role in binding DNA, the BAF57 HMG domain was dispensable for AR interaction; this interaction was primarily mediated through the N-terminal PR domain (Chapter IV). Unlike AR binding to BAF57, ER appeared to require the full-length protein for interaction (13), although specific deletion of the PR domain has not yet been tested. Combined, these data suggest a possible AR specific model wherein the PR domain docks to the AR, while the HMG domain stabilizes the association with DNA. Given the ability of the C-terminal region of BAF57 to retain SWI/SNF association (11), this region would be the likely link to SWI/SNF.

Further analyses confirmed an early recruitment of BAF57 with subsequent loss of occupancy at the PSA enhancer at later time points (Chapter IV). This finding correlates with the hypothesis that SWI/SNF is recruited to DNA (possibly through BAF57) and is able to transiently modify the chromatin and actively progress along the DNA. Therefore, although BAF57 may initially stabilize AR on DNA, the subsequent loss of BAF57 at the promoter and retention of AR, would suggest that additional factors contribute to AR stability at DNA. Alternatively, the observed “loss” of BAF57 recruitment could potentially be a result of epitope masking upon the recruitment of additional factors to the PSA enhancer region.

BAF57 Convergence on Coactivator Function

Although the mechanism by which BAF57 recruitment occurs is not fully understood, there are several key signals that are likely possibilities. One such possibility, as mentioned above, is the direct interaction with AR. Alternatively, this process could be mediated through interaction with and/or function of specific AR co-regulatory factors. In addition to its ability to bind AR and DNA, previous studies have demonstrated strong binding of BAF57 to a group of well-characterized steroid receptor coactivators (the p160 class, SRC1, TIF2, and RAC3) that have been shown to play a major role in AR transcriptional activation (9, 14). As demonstrated, BAF57 plays an important role in facilitating coactivator mediated AR activity (Chapter III). This was shown for the p160 class, as well as additional non-p160 AR coactivators (ARA70, ARA55, and Ubc9). Taken together, this would suggest a model wherein BAF57 may target the SWI/SNF

complex to the appropriate locus through a series of direct interactions with the receptor and DNA, promoting remodeling of the chromatin, which would then be followed by docking of p160 coactivators to assist in maintaining an open chromatin state. The ability of several different AR coactivators to enhance BAF57 function, suggests a more versatile role for coactivator coordination with BAF57. For example, it is not known whether or not BAF57 is capable of binding to the non-p160 coactivators examined in the present study. The minimally defined roles for these coactivators present difficulty for determining the specific impact of BAF57 on AR coactivator function. Future studies could include examining the sequential impact of specific AR coactivators in modulating BAF57 mediated function, paying close attention to the specific function of the particular coactivator. Despite not knowing the precise mechanism of BAF57 mediated coactivator activation of AR, it is clear that both factors are important for mediating the transcriptional activation of AR. Additionally, the SRC1, TIF2, and ARA70 coactivators are often overexpressed in prostate cancer tissue, highlighting the significance of these components for cancer progression (15-17). Interestingly, BAF57 expression levels, as shown in Chapter IV, demonstrate that expression levels are also retained and possibly enhanced throughout certain stages of disease, including metastatic. Together, this evidence is highly suggestive that BAF57 and specific AR coactivators contribute to the development, progression, and maintenance of prostate cancer and could potentially serve as therapeutic targets in the treatment of this disease.

Developing BAF57 as a Therapeutic Target

The initial studies demonstrated the reliance of AR signaling on BAF57 function by examining AR activity and prostate cancer cell proliferation. Upon establishing the critical importance of BAF57 in AR activation, the subsequent goal involved exploiting this subunit as a target for the treatment of prostate cancer. Expression of BAF57 in human prostate cancer tissue specimens validated this protein as a potential target. Microarray data further supported this finding wherein BAF57 mRNA expression was significantly enhanced in metastatic tissue (obtained from the oncomine database) (18). Thus, it is tempting to speculate that it would be possible to treat metastatic prostate cancer through inhibition of BAF57 function.

The data herein strongly support the development of specific means to disrupt the AR-BAF57 interaction. While the binding region of BAF57 was mapped to the AR DBD/hinge domain, directly targeting the AR (using an AR peptide) through this region may not be an effective means to disrupt AR signaling. This region is critical for modulating the binding of several AR transcriptional repressors such as SRY, FLNa, and calreticulin (19), which would possibly lend to an increase in AR signaling if this region is blocked. However, further work must be performed to challenge this speculation.

Contrary to this potential caveat, the N-terminal region of BAF57, specifically the PR domain, plays a critical role in mediating AR binding (Chapter IV), and as such, could be of significant interest as a targeted region. Given the evidence described, it is possible that BAF57 exhibits specificity of function through the PR domain. This lends to the possibility of directly targeting this

region to disrupt AR interaction and activation. Although BAF57 has been shown to play a critical role in coactivator mediated ER activation, the differences in binding to each receptor are suggestive of specificity (13). The possibility remains that side effects may result from the potentially unidentified roles of BAF57 in other tissue, and this must be directly examined by determining the impact of the AR-BAF57 binding inhibitor on other model systems; however, current observations and findings in the literature allude to the specificity of BAF57 action, strengthening its potential as a therapeutic target for the treatment of prostate cancer.

Expanding on this observation, treatment of advanced prostate cancer primarily relies on utilizing androgen receptor antagonists to block AR activity (directed at the C-terminal ligand binding domain). Additionally, no apparent role for SWI/SNF or BAF57 in antagonist mediated repression was observed (Chapter IV). Despite the selectivity of BAF57 for AR agonist function, SWI/SNF and BAF57 have both previously demonstrated repressive responses for gene expression. BRG1 and BRM interact with the transcriptional repressor prohibitin assisting in ER antagonist mediated repression. The repressive ability of BAF57 was shown through its association with the REST-Co-REST complex necessary for repression of neuronal genes (20). However, this appears to not be the mechanism for AR antagonist mediated repression, as depletion of BRM/BRG1 or inhibition of BAF57 function exhibited no impact on bicalutamide mediated repression of AR target genes (Chapter IV). The observed differences for the repressive ability of SWI/SNF and BAF57 highlight the potential mechanism

specific effects on transcriptional regulation, further supporting the possibility of selectively targeting the complex for a specific disease. Furthermore, the DBD/hinge region of AR would be the primary targeted domains. To date, no known therapeutic treatment options exist that directly target this region of AR, highlighting the novelty of this approach. Together, these data suggest that it

Figure 1 Targeting BAF57 to inhibit AR transcriptional activity. The proposed model suggests recruitment of AR to AREs upon ligand stimulation, followed by SWI/SNF recruitment through the direct interaction between AR and BAF57. Subsequent binding of co-activators is thought to assist in maintaining an open chromatin platform to allow basal transcription machinery to bind and carry out transcription on essential target genes necessary for PCa progression. The N-terminal region of BAF57 (1-145) is able to bind AR, decrease AR mediated activation of target genes and deter PCa proliferation.

may in fact be plausible to treat prostate cancer by combining BAF57 inhibition and conventional AR antagonists, thereby targeting both the DBD/hinge domain and the ligand binding domain of AR, simultaneously, in hopes of enhancing the effectiveness of prostate cancer treatment.

The presented studies demonstrate effective inhibition of AR recruitment, transcriptional activity, and prostate cancer cell proliferation through the use of a 145 amino acid peptide of BAF57 (Figure 1 and Chapter IV). These data bolster the possibility of

targeting BAF57 for the treatment of prostate cancer; however, this large peptide would obviously not be used as a therapeutic agent as is. Several possibilities exist in terms of how to go about maximizing the ability to disrupt prostate cancer proliferation through the AR-BAF57 interaction. One such possibility is to refine the AR binding motif to an even smaller region of BAF57 (if it is smaller than already determined) in hopes of generating one or several small peptide inhibitors that block BAF57 function. Additionally, utilizing the AR-BAF57 interaction as a testing medium, drug compound libraries could be screened for the specific inhibition of the AR-BAF57 interaction. Not only would it be possible to identify compounds that specifically bind BAF57 to inhibit function, but additional inhibitors could be identified that bind to the DBD/hinge domain of AR that will block BAF57 binding (presuming there is no impact on AR corepressor activity). Thus, there are several options by which to proceed in developing this as a therapeutic option.

Future Studies and Summary

It is clear that although BAF57 is a prime candidate as a target for the treatment of prostate cancer, it doesn't act alone. As is evident, the SWI/SNF complex consists of several molecules that may play specific roles in SWI/SNF mediated ATP dependent chromatin remodeling. In fact, recent studies have examined the mouse homologue of the BAF155 subunit (SRG3) and have demonstrated that this component also plays an important role in mediating AR activity (21). Given that both subunits appear to be critical for AR dependent activity, it would be interesting to determine the possibility of targeting both

subunits for the treatment of prostate cancer. Other subunits may also play a role in mediating the ability of SWI/SNF to exert its function on AR, which would suggest that other subunits may also be targeted. A more mechanistic approach would be one possible way to address this. Identification of the specific components of the SWI/SNF complex that are initially present with AR at chromatin would be valuable in determining what other subunits may be involved. Knowing this would provide a much better understanding of the role of the SWI/SNF complex in eliciting AR activation and prostate cancer proliferation. The presented work has only scratched the surface of the dynamic interplay of AR coactivator function, chromatin remodeling activity, and recruitment of the basal transcriptional machinery that are specifically involved in regulating AR mediated transcription. Future studies could incorporate examining this interplay, thereby providing a more comprehensive knowledge of how AR transactivation actually occurs, thus providing valuable information for designing innovative prostate cancer treatment options.

In summary, it is evident that targeting the AR signaling pathway is a valid and promising means for identifying and developing alternative and/or adjuvant therapies for a more effective prostate cancer treatment. The identified role of BAF57 in AR-mediated prostate cancer progression lead to the uncovering of the potential for targeting BAF57 and/or SWI/SNF as a possible means of treating prostate cancer. This emerging possibility presents the opportunity to target AR through dual mechanisms (DBD/hinge and ligand binding domain), which could lead to more effective and longer lasting treatment options.

References

1. Balk, S. P. Androgen receptor as a target in androgen-independent prostate cancer. *Urology*, 60: 132-138; discussion 138-139, 2002.
2. Feldman, B. J. and Feldman, D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*, 1: 34-45, 2001.
3. Marshall, T. W., Link, K. A., Petre-Draviam, C. E., and Knudsen, K. E. Differential requirement of SWI/SNF for androgen receptor activity. *J Biol Chem*, 278: 30605-30613, 2003.
4. Inoue, H., Furukawa, T., Giannakopoulos, S., Zhou, S., King, D. S., and Tanese, N. Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors. *J Biol Chem*, 277: 41674-41685, 2002.
5. Link, K. A., Burd, C. J., Williams, E., Marshall, T., Rosson, G., Henry, E., Weissman, B., and Knudsen, K. E. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. *Mol Cell Biol*, 25: 2200-2215, 2005.
6. Kadam, S. and Emerson, B. M. Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. *Mol Cell*, 11: 377-389, 2003.
7. Reisman, D. N., Sciarrotta, J., Bouldin, T. W., Weissman, B. E., and Funkhouser, W. K. The expression of the SWI/SNF ATPase subunits BRG1 and BRM in normal human tissues. *Appl Immunohistochem Mol Morphol*, 13: 66-74, 2005.

8. Sun, A., Tawfik, O., Gayed, B., Thrasher, J. B., Hoestje, S., Li, C., and Li, B. Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers. *Prostate*, 67: 203-213, 2007.
9. Belandia, B., Orford, R. L., Hurst, H. C., and Parker, M. G. Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. *Embo J*, 21: 4094-4103, 2002.
10. Wang, W., Chi, T., Xue, Y., Zhou, S., Kuo, A., and Crabtree, G. R. Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes. *Proc Natl Acad Sci U S A*, 95: 492-498, 1998.
11. Chi, T. H., Wan, M., Zhao, K., Taniuchi, I., Chen, L., Littman, D. R., and Crabtree, G. R. Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature*, 418: 195-199, 2002.
12. Boonyaratanakornkit, V., Melvin, V., Prendergast, P., Altmann, M., Ronfani, L., Bianchi, M. E., Taraseviciene, L., Nordeen, S. K., Allegretto, E. A., and Edwards, D. P. High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. *Mol Cell Biol*, 18: 4471-4487, 1998.
13. Garcia-Pedrero, J. M., Kiskinis, E., Parker, M. G., and Belandia, B. The SWI/SNF chromatin remodeling subunit BAF57 is a critical regulator of

- estrogen receptor function in breast cancer cells. *J Biol Chem*, 281: 22656-22664, 2006.
14. Heinlein, C. A. and Chang, C. Androgen receptor (AR) coregulators: an overview. *Endocr Rev*, 23: 175-200, 2002.
 15. Fujimoto, N., Mizokami, A., Harada, S., and Matsumoto, T. Different expression of androgen receptor coactivators in human prostate. *Urology*, 58: 289-294, 2001.
 16. Gregory, C. W., He, B., Johnson, R. T., Ford, O. H., Mohler, J. L., French, F. S., and Wilson, E. M. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res*, 61: 4315-4319, 2001.
 17. Hu, Y. C., Yeh, S., Yeh, S. D., Sampson, E. R., Huang, J., Li, P., Hsu, C. L., Ting, H. J., Lin, H. K., Wang, L., Kim, E., Ni, J., and Chang, C. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. *J Biol Chem*, 2004.
 18. Rhodes, D. R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A. M. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia*, 6: 1-6, 2004.
 19. Wang, L., Hsu, C. L., and Chang, C. Androgen receptor corepressors: an overview. *Prostate*, 63: 117-130, 2005.

20. Battaglioli, E., Andres, M. E., Rose, D. W., Chenoweth, J. G., Rosenfeld, M. G., Anderson, M. E., and Mandel, G. REST repression of neuronal genes requires components of the hSWI.SNF complex. *J Biol Chem*, 277: 41038-41045, 2002.
21. Hong, C. Y., Suh, J. H., Kim, K., Gong, E. Y., Jeon, S. H., Ko, M., Seong, R. H., Kwon, H. B., and Lee, K. Modulation of androgen receptor transactivation by the SWI3-related gene product (SRG3) in multiple ways. *Mol Cell Biol*, 25: 4841-4852, 2005.