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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusion.....	10
References.....	12
Appendices (CV).....	15
Supporting Data.....	22

INTRODUCTION

Other than agents targeting androgen receptor (AR) signaling, molecularly targeted agents have so far demonstrated limited clinical benefit in patients with castration resistant prostate cancer (CRPC). Specifically, therapeutic targets involving signaling pathways that augment or bypass AR signaling, which could be targeted either in conjunction with or upon progression from novel hormonal therapies, have yet to be fully characterized. We have performed a high throughput, *in vivo* genetic screen to identify kinases that permitted androgen-dependent transformed prostate epithelial cells (LHSR-AR cells) to form tumors in female animals. In addition to known prostate cancer oncogenes and mediators of androgen independence (mutated KRAS, constitutively active MEK, RAF1, ERBB2, AKT1, PIM1 and PIM2), overexpression of the Never In Mitosis A (NIMA) related kinase 6 (NEK6) reproducibly yielded androgen-independent tumors. NEK6 can confer castration resistance to established tumors in male mice, and inactivating its expression can restore sensitivity to castration. Castration-resistant tumors generated through NEK6 overexpression are predominantly squamous in histology and AR-negative, and NEK6 does not activate AR signaling. NEK6 overexpression leads to increased phosphorylation of two putative substrates from the literature, RPS6KB1 and SGK1, along with novel substrates identified through phosphoproteomic analysis, including FOXJ2, HUWE1 (ARF-BP1), and NCOA5. NEK6 overexpression also leads to phosphorylation of two additional forkhead-box proteins, FOXO3 and FOXA1, at proline-directed (S-P) motifs. Of the tested substrates, FOXJ2, RPS6KB1 and SGK1 are essential for NEK6-mediated androgen-independent tumor formation. NEK6 is located on a region of recurrent copy number gain on chromosome 9q33.3 in human prostate cancer; preliminary analysis of the TCGA data set reveals that NEK6 amplification or overexpression predicts for poorer outcomes, and NEK6 overexpression by immunohistochemistry in microarrays of primary tumors correlates with future development of castration-resistant disease in patients. NEK6 and its downstream effectors are thus potential novel therapeutic targets in CRPC.

BODY

Background

Prostate cancer is the second most common cause of cancer death in men, and the majority of these deaths occur in patients with metastatic castration-resistant prostate cancer (CRPC). Clinical responses to novel agents that decrease circulating androgens to below castrate levels (abiraterone – Attard *et al.*, 2009) and more potent antagonists of the androgen receptor (enzalutamide – Tran *et al.*, 2009) demonstrate that the androgen receptor (AR) signaling pathway remains critical in CRPC. However, many patients do not respond to these therapies, suggesting that alternative mechanisms for AR signaling, or other pathways for tumor survival and growth, are activated in their cancer cells. Constitutive activation of kinases such as ERBB2, MAPK, PI3K/Akt, and Src (Edwards and Bartlett, 2005b) have been implicated in mediating castration resistance both by leading to phosphorylation of AR to promote its stabilization and activation (Lin *et al.*, 2001; Yeh *et al.*, 1999; Guo *et al.*, 2006), and by potentially bypassing the need for AR signaling for tumor cell survival (Pienta and Bradley, 2006). However, inhibitors of many of these kinases have failed to demonstrate significant clinical benefit, such as in phase II trials of the Src inhibitor dasatinib (Yu *et al.*, 2009) and the ERBB2 inhibitor lapatinib (Whang *et al.*, 2011). A novel agent targeting MET and VEGFR2, cabozantinib, has demonstrated important clinical activity in metastatic CRPC (Smith *et al.*, 2013), though the relevant target substrates remain unclear as this agent has promiscuous *in vitro* activity.

A number of recent studies have demonstrated the complementarity and crosstalk between kinase signaling pathways and AR signaling. Mendiratta, *et al.* (2009) developed a gene expression signature of AR signaling, and found that decreased predicted AR activity in patient samples correlated with increased predicted Src activity. Drake, *et al.* (2012) demonstrated increased tyrosine phosphorylation in tumor samples from patients with CRPC; phosphotyrosine analysis by mass spectrometry in castration-resistant carcinomas derived from a genetically engineered mouse model identified oncogene-specific tyrosine kinase signatures, including activation of EGFR, ephrin type-A receptor 2 (EPHA2), and JAK2, along with ABL1 and SRC tyrosine kinase activation. Carver, *et al.* (2011) and Mulholland, *et al.* (2011) demonstrated cross-regulation through reciprocal feedback between the AR and PTEN/PI3K signaling pathways, suggesting combined AR and PI3K pathway inhibition as a therapeutic strategy in CRPC.

We hypothesize that novel signaling pathways may be involved in conferring castrate resistance, and given that kinases usually act as transducers of growth and proliferation signals, we hypothesize more specifically that activated/amplified kinases play a role in the development of castration resistance. We have thus performed an *in vivo*

functional genomic screen to identify novel pathways that may be involved and likely serve as therapeutic targets in these patients.

Previous work in our laboratory (Berger *et al.*, 2004) demonstrated that primary prostate epithelial cells (PrECs) that are rendered tumorigenic by the expression of the SV40 large T and small t antigens, the catalytic subunit of telomerase (hTERT), H-Ras, and the androgen receptor (LHRS-AR cells) form well-differentiated tumors in mice. These tumors are androgen-dependent and are thus unable to grow in female or castrated mice. I have performed a high throughput, *in vivo* genetic screen to identify kinases that permitted these cells to form tumors in female animals. A lentivirally delivered kinase ORF library encompassing 601 kinases and other oncogenes was introduced into these cells in pools of 9-10, and I identified fourteen ORF integrants that allowed for the androgen-independent development of subcutaneous tumors by PCR using vector specific primers (Table 1). Using the same ORF library, I performed an *in vitro* screen for genes conferring androgen-independent proliferation to androgen dependent LNCaP cells and identified 13 genes that significantly (>2 standard deviations from median) increased proliferation in androgen-deprived conditions (Table 1).

The 24 total candidates identified from both screens were introduced into LHRS-AR cells individually and injected into 6 subcutaneous sites of female BALB/C nude mice for validation of androgen-independent tumor formation. Among the candidates that reproducibly yield androgen-independent tumors are mutated KRAS; RAF1, which is recurrently translocated (Palanisamy *et al.* 2010) and amplified (Taylor *et al.*, 2010) in advanced prostate cancer; ERBB2, AKT1, and constitutively active MEK1, which have been implicated in androgen independence (Edwards and Bartlett, 2005a); and PIM1 and PIM2, which have previously been demonstrated to be important oncogenes in prostate cancer (Brault *et al.*, 2010). Among the strongest candidates identified to confer androgen independence in this assay are the Never In Mitosis A (NIMA) related kinase 6 (NEK6), and nemo-like kinase (NLK).

Aim 1. Elucidating the role of NIMA-related kinase 6 (NEK6) and nemo-like kinase (NLK) as mediators of castrate-resistant prostate cancer and assessing their potential as therapeutic targets.

Milestone 1: Determine whether NEK6 and NLK can confer castrate resistance to established tumors, whether kinase activity is required, and whether their continued expression is required.

In additional validation, NEK6 expression reproducibly and robustly yielded androgen-independent tumors, but tumor formation mediated by NLK was less robust. In addition, there is minimal evidence for alterations of NLK in human prostate cancer, so I have focused my studies on NEK6. NEK6 is overexpressed in several malignant tissues and cell lines, and has been previously been implicated in cell transformation (Jeon *et al.*, 2010; Nassirpour *et al.*, 2010). However, the mechanistic basis of its transformation phenotype and the tumor type(s) in which its activity is most relevant remain unclear. In human prostate cancer, the region encompassing NEK6 at chromosome 9q33.3 is present in a region of recurrent copy number gain (Taylor *et al.*, 2010; Huang *et al.*, 2012; Grasso *et al.*, 2012) without a known validated prostate cancer oncogene, suggesting a possible role in prostate cancer pathogenesis.

In addition to conferring tumor formation in female mice, overexpression of NEK6 in LHRS-AR cells can also lead to tumor formation in castrated mice, which lack circulating androgens since mice do not synthesize androgens from their adrenal glands (Figure 1A). The tumors in castrated mice are generally

Table 1. Results of <i>in vivo</i> and <i>in vitro</i> screens for genes conferring androgen independence, and of <i>in vivo</i> validation.					
<i>In vivo</i> LHRS-AR screen		<i>In vitro</i> LNCaP screen		<i>In vivo</i> validation	
ORF	# tumors	ORF	Fold proliferation (× median) ^b	ORF	# tumors
KRASV12+MEKDD ^a	3/3	NLK	2.09	KRASV12	6/6
ERBB2	3/3	CDK6	1.90	ERBB2	5/6
NEK6	3/3	PIM1	1.72	NEK6	5/6
RAF1	3/3	CDK4	1.55	NLK	4/6
AKT1	1/3	PIM2	1.45	MEKDD	4/6
BRD3+NEK8 ^a	1/3	STK40	1.44	AKT1	3/6
PIM2	1/3	RPS6KA2	1.40	RAF1	3/6
GK	1/3	AGK	1.40	PIM1	3/6
PFKP	1/3	TGFBR1	1.40	PIM2	1/6
PRKG2	1/3	DAPK3	1.39	others	0/6
TGFBR2	1/3	LOC389599	1.38		
PIM1	1/3	NEK5	1.37		
		AKT1	1.37		

^a Two ORF integrants were amplified from these tumors

^b For reference, the synthetic androgen R1881 leads to median 2.06 fold proliferation

smaller and slower growing than those in female mice, suggesting that signaling mediated by androgens play a role in the growth of the tumors even though they are not essential for tumor formation. To assess whether NEK6 could serve as a therapeutic target in established tumors where its activity is increased, we implanted cells with doxycycline-inducible expression of NEK6 (Figure 1B) in male mice with a testosterone pellet, and tumors were allowed to form in the presence of doxycycline in the diet. After 35 days, the mice were castrated, testosterone pellets was removed, and doxycycline was either continued or withdrawn (5 mice = 15 tumors per group; mice that died perioperatively were excluded from the analysis). As demonstrated in Figure 1C, at day 30 after castration, NEK6 confers resistance to castration compared to the parental cells when its expression is maintained with doxycycline ($p=0.001$), but sensitivity to castration is restored when doxycycline is withdrawn ($p=0.049$).

The kinase activity of NEK6 is required for conferring androgen independence, since expression of a kinase-dead form of NEK6 (K74M/K75M) did not lead to tumor formation in castrated mice (Figure 1D). The activation by its upstream kinase NEK9 (Belham *et al.*, 2003) appears to be required as mutation of the NEK9 phosphorylation site to alanine (S206A) led to decreased tumor formation. A predicted activating mutation (Y108A) through disruption of an autoinhibitory motif by homology to NEK7 (Richards, *et al.*, 2009), did not enhance tumor formation. To determine domains of NEK6 required for conferring androgen independence, 7 mutant forms with deletions of 30-40 amino acids across the length of the protein were generated. All deletions led to decreased tumor formation except deletion of amino acids 13-44, which seemed to increase tumor formation, thus demonstrating that the N-terminal is dispensable for the androgen independence phenotype.

Western blotting demonstrates increased NEK6 levels in several patient-derived prostate cancer cell lines compared with immortalized (RWPE, LH) and transformed (LHSCR-AR) prostate epithelial cells (Figure 2A). NEK6 levels are relatively high in VCaP and LNCaP cells, suggesting that high expression of NEK6 is not sufficient to overcome the *in vitro* androgen dependence of these cells. We are in the process of assessing the dependency of these cell lines on NEK6 expression by assaying viability after knockdown of NEK6 by shRNAs.

Milestone 1: NEK6 can confer castrate resistance to established tumors, kinase activity is required, N-terminal is dispensable, and continued expression is required.

Aim 2: Assessing signaling pathways involved in NEK6 and NLK-mediated castrate resistance and assessing their clinical relevance.

Milestone 2: Determine whether AR and STAT3 are necessary to confer NEK6 and NLK-mediated castrate resistance

Milestone 3: Determine impact of NEK6 and NLK on AR and STAT3 compared to other hits in the screen in vitro and in vivo

Given that persistence of AR signaling has been demonstrated to be an important mechanism of castration resistance, we sought to determine if NEK6 influences AR signaling. The tumors formed due to NEK6 overexpression have regions of nuclear AR expression but the majority of these tumors are AR-negative (Figure 3A). H&E staining demonstrates squamous differentiation in these tumors with the more mature differentiated regions demonstrating keratin deposition and AR loss. Androgen-independent tumors derived from expression of the other kinases identified in the screen also have regions of AR-positivity and negativity that vary in proportion and intensity (Supplemental Table 1.) NEK6 overexpression does not lead to an increase in activity of an AR reporter based on the PSA enhancer in LNCaP cells (Figure 3B), and inducible overexpression of NEK6 in LHSCR-AR cells (Figure 3C) does not lead to increased expression of the AR targets PSA and TMPRSS2 (Figure 3D). An mRNA expression signature of NEK6 activity was generated from cells inducibly expressing wild-type vs. kinase dead NEK6 six hours after growth factor stimulation in 3 biologic replicates collected on different days; gene expression was assayed on an Affymetrix GeneChip® Human Gene 1.0 ST Array. Gene set enrichment analysis (Subramanian *et al.*, 2005) demonstrates that gene expression changes associated with NEK6 kinase activity do not correlate positively or negatively (Figure 3E) with two previously published signatures of AR activity (Hieronymus *et al.*, 2006; Mendiratta *et al.*, 2009).

Since NEK6 does not influence AR signaling positively or negatively, we investigated other phenotypes ascribed to NEK6 in the literature. NEK6 has previously been described to play a role in the G2/M transition (Yin *et al.*, 2003), and thus we assessed whether NEK6 overexpression can influence cell cycle progression. The baseline cell cycle profile is identical for cells with inducible expression of NEK6 with and without doxycycline in normally cycling cells, cells starved in growth-factor free media, and in cells released into growth-factor containing media (Figure 4A). NEK6 does

not increase proliferation rate of LHSR-AR cells *in vitro* compared to a lacZ control (Figure 4B). Thus, the functional role of NEK6 overexpression in these cells does not appear to be related to cell cycle progression. Another activity of NEK6 that has been implicated in its oncogenic activity is inhibition of p53-mediated senescence (Jee *et al.*, 2010). LHSR-AR cells express large-T antigen, so the p53 pathway should be inactive in these cells. To confirm this in our multiply infected LHSR-AR cells, they were exposed to etoposide at 10 μ M for 18 hours or 50 μ M for 4 hours: under neither condition is p21 expression induced, while the level of cleaved PARP is not altered by NEK6 expression (Figure 4C). Thus NEK6 does not act by modulating the p53 pathway, and NEK6 overexpression is not a non-specific mediator of cell survival.

Given that NEK6 does not seem to be functioning in this system through promotion of cell cycle progression, we sought to determine whether NEK6 may exert its effect through canonical oncogenic signaling pathways. NEK6 purified from rat liver was found to be the major protein kinase that is active on the p70 S6 kinase (RPS6KB1) hydrophobic regulatory site, Thr412 (Belham *et al.*, 2001). Subsequently, it was demonstrated that NEK6 could phosphorylate hydrophobic motifs of RPS6KB1 as well as SGK1 *in vitro* (Lizcano *et al.*, 2002). However, in 293-T cells, forms of RPS6KB1 and SGK1 with mutation of the NEK6 recognition motif were phosphorylated equivalently to the wild-type forms upon stimulation by IGF-1. Thus, NEK6 did not appear to be involved in these phosphorylation events in this specific *in vivo* context.

Levels of phosphorylated Thr412 of RPS6KB1 and Ser422 of SGK1 are not different in LHSR-AR cells overexpressing wild-type NEK6 as compared to a kinase-dead control in asynchronously cycling cells in complete media, or cells starved in growth-factor free media for 36 hours (Figure 5A). However, cells overexpressing NEK6 had elevated levels of phosphorylation at these sites upon growth factor stimulation for 5 or 15 minutes compared to control. The phosphorylation of AKT1 at Ser473 is decreased with NEK6 overexpression, suggesting potential feedback inhibition of AKT1 given activation of kinases downstream of AKT1. ERK1/2 is also increased by NEK6 overexpression; however an increase of phosphorylation of STAT3 at Serine 727, which has been implicated in the transformation activity of NEK6 previously (Jeon *et al.*, 2010), was not detectable in our system using two different antibodies.

To assess whether NEK6 plays a role in cells with native high NEK6 levels not exogenously overexpressed, we suppressed NEK6 expression in DU145 cells using doxycycline-inducible shRNAs. We growth factor starved and then stimulated these cells as for the LHSR-AR cells and found that knockdown of NEK6 decreased phosphorylation at Thr412 and Ser422 of RPS6KB1 and SGK1, respectively (Figure 5B). This suggests that these two proteins are bona fide *in vivo* substrates of NEK6 in this context.

Milestones 2 and 3: NEK6 does not activate AR signaling and does not lead to detectable STAT3 phosphorylation in LHSR-AR. Rather, NEK6 phosphorylates previously identified *in vitro* substrates RPS6KB1 and SGK1 in response to growth factor stimulation.

Milestone 4: Generation of mRNA and phosphoproteomic signatures corresponding to androgen independence conferred by our hits, and comparison to androgen signaling and existing databases

To assess downstream signaling pathways activated by NEK6 overexpression, we utilized the R&D Systems Human Phospho-Kinase Antibody Array to compare with signaling mediated by overexpression of two other kinases identified in our initial screen involved in canonical oncogenic signaling pathways, AKT1 and RAF1. Lysates were obtained from cells expressing doxycycline-inducible wild-type NEK6 and kinase-dead NEK6, along with AKT1 and RAF1, 1 hour after growth factor stimulation. The patterns of downstream signaling were compared to kinase-dead NEK6 as a control. The profile for wild-type NEK6 overexpression is nearly identical to that mediated by AKT1 overexpression, except for a decrease in phosphorylation of AKT at Ser473 and Thr308 with NEK6 overexpression (Figure 5C). Again, this suggests that NEK6 activates similar downstream signaling as AKT1 with feedback inhibition of AKT1 itself. Phosphorylation of Thr412 of RPS6KB1 and Ser422 of SGK1 are not assayed in this array. RAF1 overexpression leads to some overlapping signal with wild-type NEK6 and AKT1, but not the same breadth of changes as the others.

To gain a more comprehensive understanding of the immediate signaling mediated by NEK6 expression and discover novel *in vivo* substrates, we collaborated with the Proteomics Platform at the Broad Institute of Harvard and MIT for phosphoproteomic analysis. Constructs for the expression of wild-type and kinase-dead NEK6 under the control of a doxycycline-inducible promoter were introduced into LHSR-AR cells; phosphoproteomic profile of cells expressing wild-type NEK6 induced by doxycycline were compared to those of cells cultured in the absence of doxycycline and cells

expressing kinase-dead NEK6 induced by doxycycline. Cells were cultured in “light”, “medium”, and “heavy” medium for Stable Isotope Labeling by Amino acids in Cell culture (SILAC) for 7 days in 2 different permutations; they were growth factor starved for the final 30 hours and then growth factor stimulated for 5 minutes as in Figure 5A. Lysates were then subjected to SCX-IMAC phosphorylation analysis and assayed by mass spectrometry.

A total of 9418 phosphopeptides (8432 phosphoserine, 952 phosphothreonine, 34 phosphotyrosine) from 3401 proteins were detected in this experiment. 59 phosphopeptides from 50 proteins were increased in both the wild-type induced vs. un-induced and wild-type vs. kinase dead comparison with a combined q value of <0.25 (Supplemental Table 2). The differentially phosphorylated proteins include a large number of transcriptional regulators, including the forkhead-box family proteins FOXO3 and FOXA1, and the phosphopeptides represent a variety of common phosphorylation motifs as shown in Table 2. Among these motifs are pS/pT-P, associated with MAPK/CDK/GSK3 signaling, and R-X-R-X-X-pS/pT, associated with AKT and RSK (ribosomal S6-kinase) signaling. However, these motifs are not overrepresented in the phosphopeptides enriched with wild-type NEK6 expression. Immunoblotting with motif-specific antibodies for MAPK/CDK and AKT/RSK signaling demonstrates an increased intensity of only specific bands with NEK6 overexpression (Figure 6A), demonstrating that NEK6 is not a global activator of these signaling pathways.

Table 2. Summary of phosphoproteomic data

	L-X-X-pS/pT-F/W/Y/M/L/I/V/R/K (NEK6 general consensus)	L/F/W/Y-X-X-pS/pT-F/W/Y/M/L/I/V/R/K (NEK6 acceptable)	pS/pT-P (proline-directed kinases)	R-X-R-X-X-pS/pT (AKT1 or RSK family)	R-X-X-pS/pT (CaMK consensus)	pS/pT-D/E-X-D/E (CK2 consensus)	Others
Proteins with phosphopeptides meeting significance threshold (59 total phosphopeptides representing 50 proteins)	FOXJ2 HUWE1 NCOA5 KRT18	FOXJ2 HUWE1 NCOA5 KRT18 TRA2B (Y-X-X-pS-Y) HNRNPM (F-X-X-pS-F) HNRNPA2B1 (F-X-X-pS-F) ZNF326 (F-X-X-pS-Y)	FOXO3 TRPS1 LMO7 SATB2 PAK6 BCL6 FOXA1 KLF4 ATXN1 IRF2BP1 RFX2 HIVEP2 KLF3 Others (×13)	SLC2A12 FAM21C ATXN1	SLC2A12 FAM21C ATXN1 ATM LMO7 DROSHA PLEKHA6 RIPK3 MYOF EXPH5 MLLT3	ERCC5 LIG1 PBRM1	16 phosphopeptides representing 14 proteins 12 pS 3 pT 1 pY
Enrichment (compared to all detected phosphopeptides)	4/59 vs. 131/9362 (p=0.0092 Fisher's exact)	8/59 vs. 186/9362 (p=1.95×10 ⁻⁵)	26/59 vs. 4815/9434 (p=NS)	3/59 vs. 434/9301 (p=NS)	11/59 vs. 2239/9362 (p=NS)	3/59 vs. 701/9417 (p=NS)	

A canonical NEK6 motif (Vaz Meirelles *et al.*, 2010) with leucine at the -3 position [L-X-X-pS/pT-F/W/Y/M/L/I/V/R/K] is more frequently represented in the 59 significantly enriched phosphopeptides compared to all detected phosphopeptides (p=0.0092 Fisher's exact); an “acceptable” NEK6 substrate motif [L/F/W/Y-X-X-pS/pT-F/W/Y/M/L/I/V/R/K] is even more significantly enriched (p=1.95×10⁻⁵). This suggests that this peptide sequence is a true description of the NEK6 phosphorylation motif, and that the 8 proteins with phosphopeptides of this form detected here (FOXJ2, HUWE1, NCOA5, KRT18, TRA2B, HNRNPM, HNRNPA2B1, ZNF326) are bona fide *in vivo* NEK6 substrates.

To confirm whether detection of these phosphopeptides in cells is due to direct phosphorylation by NEK6, we assayed the kinase activity of commercially available recombinant enzyme on a subset of these proteins expressed with a C-terminal V5-tag. We were able to confirm phosphorylation of two proteins identified in our phosphoproteomic experiment, FOXJ2 and NCOA5, with no phosphorylation of a lacZ control (Figure 6B). Mutation of the phosphorylation sites of FOXJ2 and NCOA5 identified via mass spectrometry, Ser8 and Ser96 respectively, decreases the *in vitro* phosphorylation of these substrates, demonstrating the specificity of the kinase activity at these sites.

To understand which of these substrates may play a role in NEK6-mediated androgen independent tumor formation, we designed constructs for inducible suppression of the expression of five putative substrates (two from the literature and three from Table 2) with two different short hairpin RNAs (Figure 7A). LHSR-AR cells expressing NEK6 and one of these inducible shRNAs were implanted into three subcutaneous sites of female nude mice fed doxycycline in their diet. As expected, inducible expression of an shRNA targeting NEK6 itself dramatically decreased tumor formation as compared to a control shRNA targeting lacZ (Figure 7B). In addition, suppression of FOXJ2, RPS6KB1 and SGK1 also dramatically decreased tumor formation mediated by NEK6 overexpression, while knockdown of HUWE1 and NCOA5 did not. We are in the process of determining if these mediators are also essential in androgen-independent tumor formation mediated by a different oncogene in our model, constitutively active MEK, which primarily signals through the MAP kinase pathway. In addition, we are testing a pharmacologic inhibitor of SGK1 for inhibiting androgen-independent tumor formation mediated by NEK6 overexpression.

Milestone 4: mRNA expression signature of NEK6 activity demonstrates no positive or negative correlation with AR signaling. Phosphoproteomic signature reveals novel substrates FOXJ2, HUWE1, NCOA5, KRT18, TRA2B, HNRNPM, HNRNPA2B1, ZNF326 along with phosphorylation of FOXO3 and FOXA1 at pS/pT-P sites. NEK6 substrates FOXJ2, RPS6KB1 and SGK1 are required for NEK6-mediated androgen independent tumor formation.

Milestone 5: Determine evidence for NEK6 and NLK conferring castrate resistance in clinical samples and identify subsets of patients displaying genomic changes and expression signatures corresponding to resistance mediated through the activity of a particular gene/pathway.

To assess if high NEK6 expression is associated with more aggressive disease in patients, we assayed NEK6 expression in tumor microarrays of primary localized prostate cancer. Primary prostate cancer is a heterogeneous disease with a mixture of more and less malignant clones; we would hypothesize that foci of high NEK6 expression in primary tumors may represent more malignant clones and that patients with these foci would be more likely to develop castration-resistant disease than patients without these foci. The number of events (recurrences, development of metastatic/castration resistant disease) in this cohort is not large enough for separate training and validation sets; thus the threshold designating “high” NEK6 expression was set at a level best differentiating tumor and benign prostate tissue across the whole data set. High NEK6 expression by IHC does not correlate with Gleason grade (Figure 8). However, patients with high NEK6 levels have a higher incidence of development of metastatic disease and castration resistant disease (Figure 9A). These patients also have inferior 5 year biochemical recurrence (BCR)-free survival (Figure 9B), though the difference between the Kaplan-Meier curves does not reach statistical significance in this data set (median time to BCR for NEK6 low=not reached, NEK6 high=10 months, $p=0.09$). These findings are corroborated in the current provisional TCGA dataset available on the cBioPortal (<http://www.cbioportal.org/public-portal/index.do>) where cases demonstrating NEK6 amplification or overexpression have worse disease-free survival (log rank $p = 0.0018$) compared to other cases (Figure 9C). These findings would need to be confirmed in larger multi-institutional cohorts to be validated as a clinically useful prognostic marker, but these findings suggest that high NEK6 expression is a marker of more aggressive disease. The potent phenotype mediated by NEK6 overexpression in our model suggests a mechanistic relationship to the development of lethal disease in these patients.

It remains unclear at this point whether *de novo* acquisition of NEK6 amplification or overexpression is a mechanism of development of castration resistance in advanced prostate cancer. The strongest evidence for this would be detection of markers of overactive NEK6 signaling in metastatic CRPC in comparison to therapy-naïve samples, which per our model would be most likely to be detected in prostate cancers that have become AR-independent. A recent study by Grasso *et al* (2012) genetically profiled 35 cases of CRPC in comparison to 59 cases of localized PrCa. The primary difference in the genomic landscape in these two states was massive amplification of the AR in CRPC in comparison to the primary cases; there was no increase in amplitude of the region of copy number gain on 9q33.3 where NEK6 is located. There was no evidence for enrichment in genetic markers of any other signaling pathways in CRPC cases, though this is likely due to the fact that these cases were collected at a time when many of the current novel hormonal therapies had not yet been in widespread use. Efforts to genetically characterize larger numbers of CRPC cases, including those from patients who have progressed on novel hormonal therapies, are currently underway at DFCI (<http://www.aacr.org/home/public--media/stand-up-to-cancer/su2c-dream-teams/su2c-pcf-dream-team-precision-therapy-of-advanced-prostate-cancer.aspx>).

Milestone 5: NEK6 overexpression in primary tumors predicts for more aggressive disease. Efforts to characterize metastatic biopsies from patients with CRPC for sequencing and assessing gene expression are underway.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of NEK6 as a novel mediator of castration resistance in an *in vivo* forward functional genomic screen.
- Demonstration that kinase activity and continued expression of NEK6 is required for maintenance of castration resistance, suggesting its suitability as a therapeutic target.
- Confirmation of previously identified *in vitro* substrates RPS6KB1 and SGK1 as bona fide *in vivo* substrates, and discovery of novel NEK6 substrates FOXJ2, HUWE1, NCOA5, KRT18, TRA2B, HNRNPM, HNRNPA2B1, ZNF326.
- Demonstration that substrates FOXJ2, RPS6KB1 and SGK1 are required for NEK6-mediated androgen-independent tumor formation.
- Determination that high NEK6 expression in primary tumors predicts for more aggressive disease

REPORTABLE OUTCOMES

Research Investigations

Choudhury AD, Lock YJ, Guney I, Pei T, Schinzel AC, Izzo F, Lis R, Stack EC, Nakabayashi M, Petrozziello G, Patel J, Jaffe JD, Kantoff PW, Loda M, Hahn WC. "An *in vivo* functional genomic screen identifies NEK6 as a novel mediator of castration resistance in prostate cancer." (manuscript in progress)

Reviews

Choudhury AD, Eeles R, Freedland SJ, Isaacs WB, Pomerantz MM, Schalken JA, Tammela TL, Visakorpi T. "The role of genetic markers in the management of prostate cancer." *Eur Urol.* 2012 Oct;62(4):577-87.

Choudhury AD, Kantoff PW. "New Agents in Metastatic Prostate Cancer". *J Natl Compr Canc Netw.* 2012 Nov 1;10(11):1403-9.

Abstracts, Poster Presentations and Exhibits Presented at Professional Meetings

Abstracts

Choudhury AD, Guney I, Schinzel AC, Izzo F, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." 18th Annual Prostate Cancer Foundation Scientific Retreat, September 21, 2011, Young Investigator Presentation.

Poster Presentations

Choudhury AD, Guney I, Schinzel AC, Izzo F, Stack EC, Nakabayashi M, Petrozziello G, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." 5th Annual Multi-institutional Prostate Cancer Program Retreat, March 19-21, 2012. Poster selected for presentation and awarded as a prize winner.

Choudhury AD, Guney I, Schinzel AC, Izzo F, Stack EC, Nakabayashi M, Petrozziello G, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." Dana-Farber Cancer Institute Molecular and Cellular Oncology Department Retreat. April 9, 2012.

CONCLUSIONS

While there has been significant recent progress in the treatment of metastatic castration-resistant prostate cancer (Choudhury and Kantoff, 2012), the clinical benefit from molecularly targeted agents in CRPC other than for those that target androgen receptor signaling remains limited. This is likely because our understanding of therapeutic targets in CRPC is incomplete and patients have not been successfully stratified for the likelihood of response to a given therapy. We have performed an unbiased *in vivo* functional genomic screen to identify genes that can confer androgen independence in a model of androgen-dependent prostate tumor formation, and discovered NEK6 as a novel mediator of castration resistance. The NEK6 gene on 9q33.3 is located on a region of recurrent copy number gain in prostate cancer, and NEK6 amplification or overexpression in primary prostate cancer predicts for poor outcomes independent of grade.

NEK6 plays a mechanistic role in the development of castration resistance in our model, and turning off its expression in xenograft tumors where it is overexpressed restores sensitivity to castration, suggesting that its continued activity is required for tumor maintenance in this context. These findings suggest NEK6 as a novel therapeutic target in patients with castration-resistance mediated by NEK6 activity.

NEK6 has been previously described to be required for the mitotic progression of human cells (Yin *et al.*, 2003), and would thus be implicated as an essential gene in proliferating cells. However, a subsequent study (Nassirpour *et al.*, 2010) demonstrated that knocking down endogenous Nek6 levels or exogenous expression of the kinase-dead form in normal fibroblast cells did not inhibit cell proliferation or induce apoptosis. Thus, pharmacologic targeting of NEK6 would be predicted to be toxic only to those cells with a specific dependency. We have demonstrated that RPS6KB1 and SGK1 are *in vivo* substrates of NEK6, and identified novel substrates including FOXJ2, HUWE1 and NCOA5.

Parallel growth factor signaling has been implicated in androgen independence in several model systems, but this phenotype depends on both the growth factor milieu of the microenvironment and the underlying genetic context of the cancer cells with regards to their behavior in this milieu. The use of engineered cell lines allows control over the genetic context, and the use of xenografts allows testing different *in vivo* environments where the limiting nutrients and growth factors are unknown and thus could not be replicated in a culture environment. The apparent biologic relevance of NEK6 in human prostate cancer suggests the validity of the model system used in our original screen, though it is reasonable to consider that other genes might be identified to confer androgen independence in different genetic contexts in different microenvironments. NEK6 activity is increased by growth factor stimulation in our experiments, and identification of the specific molecules involved would provide important insight into the role of the microenvironment in activating the NEK6 pathway.

Alterations in the AR gene and in AR-mediated signaling have already been demonstrated to play a role in the development of castration resistance (Edwards and Bartlett, 2005a), and our initial screen identified several kinases that can confer androgen-independent tumor formation in our model system. It is likely that in addition to these, genes in other families (transcription factors, epigenetic modifiers, non-coding RNAs) could also lead to castration resistance. Given the multitude of potential mechanisms for development of castration-resistant disease in human prostate cancer, targeting the resistant clone(s) requires understanding the operant mechanism(s) in a particular patient. It is unclear at this point whether single molecular markers would be adequate for this purpose, or whether a combination of genetic/epigenetic, gene expression and/or phosphoproteomic features may be more informative (Choudhury, Eeles, *et al.*, 2012). It is thus essential to collect tissue from patients with advanced PrCa to discover molecular features indicating activity of a particular gene/pathway, and to assess the utility of these features in predicting responses to targeted therapies in clinical trials.

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APPENDICES (CV)

Date Prepared: July 31, 2013
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Place of Birth: Dhaka, Bangladesh

Education

1998	BA (Phi Beta Kappa Honor Society as a Junior; General and Departmental Honors)	Chemistry	Johns Hopkins University, Baltimore MD
2005	PhD	Integrated Program in Cellular, Molecular and Biophysical Studies (Richard Baer)	Columbia University Graduate School of Arts and Sciences, New York NY
2006	MD (Alpha Omega Alpha Honor Society)		Columbia University College of Physicians and Surgeons, New York NY

Postdoctoral Training

06/06-06/08	Resident	Internal Medicine	Brigham and Women's Hospital, Boston MA
07/08-06/12	Clinical Fellow	Medical Oncology (William Hahn)	Dana-Farber Cancer Institute, Boston MA

Faculty Academic Appointments

07/12-current	Instructor	Medicine	Harvard Medical School
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Appointments at Hospitals/Affiliated Institutions

07/12-current		Medical Oncology	Dana-Farber Cancer Institute, Boston MA
07/12-current		Medical Oncology	Brigham and Women's Hospital, Boston MA

Other Professional Positions

Major Administrative Leadership Positions

Local

Regional

National and International

Committee Service

Local

Regional

National and International

Professional Societies

2009-present American Association for Cancer Research
2010-present American Society of Clinical Oncology

Grant Review Activities

Editorial Activities

Other Editorial Roles

Honors and Prizes

1995 – 1998	Dean’s List (all six semesters)	Johns Hopkins University
1997	Howard Hughes Summer Research Fellowship	
1997	Phi Beta Kappa Honor Society (as junior)	
1998	General and Departmental Honors	Johns Hopkins University
2006	Alpha Omega Alpha Honor Society	

Report of Funded and Unfunded Projects

Funding Information

Past

- 08/09-08/11 Molecular Determinants of Hormone Refractory Prostate Cancer
Prostate Cancer Foundation Young Investigator Award
PI (\$150,000)
The goal of this study is to investigate the molecular mechanisms for transition from androgen-dependent to androgen-independent growth in human prostate cancer by studying several candidates that were identified through a cDNA library screen for kinases conferring androgen independence in a mouse xenograft model of prostate cancer.
- 07/09-06/12 NIH T32 National Research Service Award (NRSA) Institutional Research Training Grant
2T32CA009172-37
- 07/09-06/12 Anne Huber Foster Fellowship

Current

- 07/12-07/17 Molecular Determinants of Hormone Refractory Prostate Cancer
Department of Defense Prostate Cancer Research Program Physician Research Training
Award W81XWH-12-1-0062
PI (\$650,000)
The goal of this study is to investigate the molecular mechanisms for transition from androgen-dependent to androgen-independent growth in human prostate cancer by studying several candidates that were identified through a cDNA library screen for kinases conferring androgen independence in a mouse xenograft model of prostate cancer.

Current Unfunded Projects

- 05/11-
current Co-PI, Purification and whole exome sequencing of circulating tumors cells from patients with prostate cancer.
My role in this project is to obtain samples for CTC isolation from patients participating in clinical trials at Dana-Farber and to help in optimizing technologies for CTC isolation and sequencing (using the Illumina Magsweeper platform), while coordinating the comparison with standard techniques for CTC isolation (Veridex).

Report of Local Teaching and Training

Teaching of Students in Courses

Formal Teaching of Residents, Clinical Fellows and Research Fellows (post-docs)

Clinical Supervisory and Training Responsibilities

Laboratory and Other Research Supervisory and Training Responsibilities

7/12-current Supervising technician Jane Lock, training in basic molecular biology techniques, mouse husbandry, subcutaneous xenograft implantations

Formally Supervised Trainees

Formal Teaching of Peers (e.g., CME and other continuing education courses)

7/12-current Case discussion and literature review for Genitourinary Management and Assessment Pathway meetings

Local Invited Presentations

Report of Regional, National and International Invited Teaching and Presentations

Invited Presentations and Courses

Regional

National

5/6-5/8/13 “Molecular Determinants of Hormone-Refractory Prostate Cancer.” Biomarkers & Diagnostics World Congress, Philadelphia PA (cancelled)

International

Report of Clinical Activities and Innovations

Current Licensure and Certification

Licensure:

Exp. Date Commonwealth of Massachusetts Board of Registration in Medicine
10/07/2013

Certification:

08/2009 Internal Medicine, American Board of Internal Medicine
11/2011 Medical Oncology, American Board of Internal Medicine

Practice Activities

Clinical Innovations

Report of Technological and Other Scientific Innovations

Molecular Determinants Associated With Prostate Cancer And Methods Of Use Thereof	US Patent Application, 20110206689, filed August 25, 2011 The present invention provides methods of treating cancer by inhibiting serine threonine kinase activity and detecting cancer using biomarkers. A cDNA library screen for kinases conferring androgen independence in a mouse xenograft model of prostate cancer identified 16 kinases conferring castrate-resistant tumor formation. Six of these genes fall in regions of gene amplification in metastatic prostate tumors and cell lines: TK1, HGS, PLAU, SKP2, TNK2 and UCK2. Inhibition of the activity or expression of these kinases could serve as a strategy for treatment of castrate resistant prostate cancer (CRPC), and measurement of their expression or activity could serve as a biomarker for increased risk for developing CRPC.
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Report of Education of Patients and Service to the Community

Activities

Educational Material for Patients and the Lay Community

Books, monographs, articles and presentations in other media

Educational material or curricula developed for non-professional students

Patient educational material

Recognition

Report of Scholarship

Publications

Peer reviewed publications in print or other media

Research Investigations

Choudhury AD, Xu H, Modi AP, Zhang W, Ludwig T, Baer R. "Hyperphosphorylation of the BARD1 tumor suppressor in mitotic cells." *J Biol Chem.* 2005 Jul 1;280(26):24669-79.

Choudhury AD, Xu H, Baer R. "Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression." *J Biol Chem*. 2004 Aug 6;279(32):33909-18.

Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD, Memeo L, Mansukhani M, Murty VV, Gaciong Z, Meek SE, Piwnica-Worms H, Hibshoosh H, Parsons R. "Lack of PTEN sequesters CHK1 and initiates genetic instability." *Cancer Cell*. 2005 Feb;7(2):193-204.

Xu XZ, Choudhury A, Li X, Montell C. "Coordination of an array of signaling proteins through homo- and heteromeric interactions between PDZ domains and target proteins." *J Cell Biol*. 1998 Jul 27;142(2):545-55.

Reviews

Choudhury AD, Eeles R, Freedland SJ, Isaacs WB, Pomerantz MM, Schalken JA, Tammela TL, Visakorpi T. "The role of genetic markers in the management of prostate cancer." *Eur Urol*. 2012 Oct;62(4):577-87.

Choudhury AD, Kantoff PW. "New Agents in Metastatic Prostate Cancer". *J Natl Compr Canc Netw*. 2012 Nov 1;10(11):1403-9.

Non-peer reviewed scientific or medical publications/materials in print or other media

Professional educational materials or reports, in print or other media

Clinical Guidelines and Reports

Thesis

Choudhury, AD. "Cell cycle regulation of BRCA1 and BARD1." *Columbia University*, 2005.

Abstracts, Poster Presentations and Exhibits Presented at Professional Meetings

Abstracts

Choudhury AD, Guney I, Schinzel AC, Izzo F, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." 18th Annual Prostate Cancer Foundation Scientific Retreat, September 21, 2011, Young Investigator Presentation.

Poster Presentations

Choudhury AD, Guney I, Schinzel AC, Izzo F, Stack EC, Nakabayashi M, Petrozziello G, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." 5th Annual Multi-institutional Prostate Cancer Program Retreat, March 19-21, 2012. Poster selected for presentation and awarded as a prize winner.

Choudhury AD, Guney I, Schinzel AC, Izzo F, Stack EC, Nakabayashi M, Petrozziello G, Hahn WC. "Molecular Determinants of Hormone Refractory Prostate Cancer." Dana-Farber Cancer Institute Molecular and Cellular Oncology Department Retreat. April 9, 2012.

Narrative Report (limit to 500 words)

- I have just completed my first year as Clinical Instructor in Medical Oncology at Dana-Farber Cancer Institute, and am completing my fourth year as a post-doctoral fellow in the laboratory of Dr. William Hahn. I see patients one half day a

week in clinic, and approximately 90% of my time is devoted to basic science research. The goal of my research project is to investigate molecular mechanisms of castration resistance in prostate cancer. To this end, I have performed unbiased functional genomic screens to identify novel genes that can confer androgen independence in an *in vivo* model of androgen dependent prostate cancer. I have identified two kinases, NEK6 and NLK, which robustly and reproducibly confer androgen-independent tumor formation to androgen-dependent prostate cancer cells *in vivo*, and are thus likely to activate signaling pathways that are relevant for conferring castrate resistance in patients. This research plan has earned me a Young Investigator Award from the Prostate Cancer Foundation and a Physician Scientist Training Award from the Department of Defense, while a poster presentation of my results was one of the prize winners at the 5th Annual Multi-institutional Prostate Cancer Program Retreat.

My training program at Dana Farber Cancer Institute and my involvement with the Broad Institute of MIT and Harvard has me uniquely positioned to apply novel experimental approaches to address rapidly evolving clinical problems in prostate cancer. During my clinical training, I have cared for many cancer patients with a variety of common and uncommon diagnoses. I have thus developed an understanding of important clinical problems in medical oncology and areas of unmet clinical need from early in diagnosis to the end of life. One such area of clinical need amenable to scientific inquiry is the development of castration resistance in metastatic prostate cancer, and this problem forms the basis of my current research interests.

I have already helped facilitate collaborations between the clinical genitourinary oncology group at DFCI and the Broad Institute, specifically in identifying clinical samples being obtained through clinical trials being performed at DFCI that would be available for gene expression and next-generation sequencing studies at the Broad. I have coordinated efforts at isolation and sequencing of circulating tumor cells from patients with prostate cancer, and I have also been collaborating with Dr. Cory Johannessen and others in bringing together various disparate experimental approaches and novel technologies to study mediators of resistance to therapy in prostate cancer with the goal of establishing a "Resistance Platform".

My career goal is to attain an independent academic faculty position that will allow me to spend 90% of my time pursuing independent laboratory investigations in prostate cancer, while seeing genitourinary cancer patients approximately one half day each week. I hope to continue to study mediators of prostate cancer progression and resistance to therapy in order to discover novel targets for therapeutic intervention. Eventually, I hope to be able to apply results of my laboratory research to translational studies and therapeutic clinical trial designs.

SUPPORTING DATA

Figure 1. NEK6 confers androgen-independent tumor formation in a xenograft model of androgen-dependent prostate cancer. A. Tumor formation at 60 days for parental LHSR-AR cells and cells expressing NEK6 in female and castrated mice. B. Inducible expression of NEK6 *in vitro* at 48 hours after addition of doxycycline. C. Waterfall plot of change in tumor volume of parental LHSR-AR cells and cells with inducible NEK6 expression formed in male mice, 30 days after castration and removal of testosterone pellet. D. Tumor formation in castrated male mice mediated by expression of wild-type NEK6 (wt) and the following mutants: kinase dead (K74M/K75M), predicted constitutively active (Y108A), NEK9 activation site mutant (S206A), and deletions of the amino acids indicated.

Figure 2. NEK6 is overexpressed in several prostate cancer cell lines compared to immortalized (RWPE, LH) and transformed (LHSR-AR) prostate epithelial cells. A. Expression of NEK6 and AR in prostate cell lines with Hsp90 as loading control

Figure 3. NEK6 does not confer androgen-independent tumor formation through activation of AR. A. NEK6-mediated androgen-independent tumors are primarily squamous in histology and AR negative. Sections of tumors derived from parental LHSR-AR cells expressing GFP in male mice, and cells expressing NEK6 in female and castrated mice were stained with AR antibody (brown). B. Luciferase activity detected in LNCaP cells transiently transfected with an AR reporter either alone (top) or in combination with an expression plasmid for NEK6 (bottom) incubated with concentrations of the synthetic androgen R1881 indicated. C. Doxycycline-inducible expression of NEK6 wild-type, kinase dead (K74M/K75M), AKT1 and RAF1 both untagged and with C-terminal V5 tag. Untagged versions were used for experiments described. D. Inducible expression of NEK6 does not increase expression of AR targets PSA or TMPRSS2 in LHSR-AR cells. Expression of TMPRSS2 and PSA were measured by qPCR in the absence and presence of doxycycline to induce transgene expression and in the presence and absence of R1881 as indicated, with expression normalized to cells transduced with NEK6 K74M/K75M in the absence of doxycycline and R1881. E. NEK6 expression has neither a positive or negative effect on AR signaling as measured through published AR signatures. Gene expression changes conferred by inducible expression of wild-type NEK6 vs. kinase dead NEK6 six hours after growth factor stimulation were assayed in 3 biological replicates, and GSEA was used to assess enrichment of signatures positively correlated with AR activity in two data sets from the literature (Hieronymus *et al.*, 2006; Mendiratta *et al.*, 2009).

Figure 4. NEK6 overexpression does not lead to promotion of cell cycle progression or antagonism of the p53 pathway in LHSR-AR cells. A. Cell cycle profiles of cells with and without NEK6 overexpression. LHSR-AR cells transduced with doxycycline-inducible NEK6 were cultured in the presence (bottom profiles) or absence (top profiles) of doxycycline, starved from growth factors for 24 hours, and released into growth factor-containing media for the times indicated, then harvested and fixed for propidium iodide staining in comparison to asynchronously cycling cells. B. Proliferation curves of LHSR-AR constitutively expressing lacZ or NEK6, average cell counts from 3 plates collected at the indicated time points plotted compared to previous time point with standard deviations. C. p53 pathway is inactive in LHSR-AR cells, and NEK6 expression does not rescue from cell death mediated by etoposide. LHSR-AR cells with constitutive expression (pLX304-) of lacZ vs. NEK6 or doxycycline-inducible expression (pTRIPz-) of NEK6 kinase dead (kd) vs. wild-type (wt) were exposed to etoposide at concentrations indicated vs. DMSO as vehicle control. Attached and floating cells were harvested and combined for immunoblotting.

Figure 5. Kinase signaling mediated by NEK6. A. Overexpression of NEK6 leads to phosphorylation of published substrates RPS6KB1 and SGK1, with decreased phosphorylation of AKT1. LHSR-AR cells with doxycycline-induced expression of NEK6 kinase-dead (kd) vs. wild-type (wt) were starved of growth factors for 24 hours, then stimulated with growth factor-containing media for the indicated periods of time and harvested. B. NEK6 knockdown decreases RPS6KB1 and SGK1 phosphorylation in DU145 cells. DU145 cells with doxycycline-inducible expression of shRNAs targeting lacZ or NEK6 were starved of serum for 24 hours, then stimulated with growth factor-containing media for the indicated periods of time and harvested. C. NEK6 leads to similar downstream signaling as AKT1 with decrease in phosphorylation of AKT1 itself. LHSR-AR cells with doxycycline-inducible expression of NEK6 wt, NEK6 kd, AKT1 and RAF1 were starved of growth factors for 24 hours, then stimulated with growth factor-containing media for 1 hour. Cell lysates were incubated with R&D Systems Phospho-Kinase Array, and mean intensity of dots in duplicate were compared to NEK6 kd as control. 46 phosphorylation events were assayed; events that increased or decreased in intensity more than 1.5-fold in the comparisons of NEK6 wt (blue bars), AKT1 (yellow bars) and RAF1 (purple bars) vs. control are plotted (fewer events are plotted for RAF1 as fewer events met the fold-change thresholds described).

Figure 6. A. NEK6 overexpression increases phosphorylation of certain proteins with MAPK/CDK, AKT/RSK motifs. LHSR-AR cells with doxycycline-induced expression of NEK6 kinase-dead (kd) vs. wild-type (wt) were starved of growth factors for 24 hours, then stimulated with growth factor-containing media for the indicated periods of time and harvested; lysates were immunoblotted with the indicated motif-specific antibodies. Green arrows indicate phosphoproteins increased in intensity with expression of wild-type vs. kinase dead NEK6. B. NEK6 can phosphorylate NCOA5 and FOXJ2 *in vitro* at the sites discovered in the phosphoproteomic screen. 293T cells were transfected with expression constructs for wild-type and mutant (S-to-D) versions of NCOA5 or FOXJ2 with a C-terminal V5 tag and immunoprecipitated with anti-V5 antibody. Eluates from 1/5 of the beads were assayed by V5 immunoblot; the remaining 4/5 was subjected to on-bead *in vitro* kinase assay with recombinant active GST-NEK6 (Sigma).

Figure 7. Requirement of NEK6 substrates for NEK6-mediated androgen-independent tumor formation. A. Inducible knockdown of NEK6 substrates as assayed by qPCR in LHSR-AR cells constitutively expressing NEK6 with doxycycline-inducible expression of shRNAs; average of 2 replicates shown. B. Tumor formation in female mice of cells assayed in A.

Figure 8. NEK6 expression does not correlate with grade. Tumor microarrays representing 244 cases of primary localized prostate cancer, 215 of which were evaluable and 208 with clinical follow-up were stained with NEK6 antibody for immunohistochemistry and analyzed by CRi spectral imager. Representative images from tumor microarrays demonstrating: A,B: high NEK6, low grade. C,D: high NEK6, high grade. E,F: low NEK6, low grade. G,H: low NEK6, high grade.

Figure 9. NEK6 overexpression is correlated with inferior relapse-free survival, more metastasis and castration-resistant disease. A. Contingency tables representing proportion of NEK6 high patients (n=40) and NEK6 low patients (n=168) with different Gleason grades, recurrent vs. non-recurrent disease, metastatic vs. non-metastatic disease, and hormone-refractory vs. sensitive disease during clinical follow-up. B. Kaplan-Meier curve of biochemical relapse-free survival of NEK6 high (blue) vs. NEK6 low (red) groups in tumor microarray studies. C. Kaplan-Meier curve of disease free survival in NEK6 overexpressed (relative mRNA expression >2.0) or amplified (red) vs. other cases (blue) in preliminary TCGA data set.

Supplemental Table 1.

		AR Nuclear % score (0,1,2,3,4) ^a	AR Nuclear intensity ^b	AR % Cytoplasm score (0,1,2,3,4) ^a	AR Cytoplasm intensity ^b	notes
Male mice (positive control)	GFP 1 (one piece)	4	m s	3	w m	9.11.12
	GFP 1 (second piece)	3	w m s	1	w	
	GFP 2	4	m s	1	w	
	GFP3	4	s	4	m s	
Female mice	NEK6 1 (one piece)	2	m s	2	w m	
	NEK6 2 (few pieces)	1	w	1	w	
	NEK6 3	trace	most nuclei are neg; occasional nuclei show weak staining (<1-2%)	trace	most cytoplasm are neg; occasional cells show weak staining (<1-2%)	surface mouse skin is neg for AR
	AKT1	2	m s	3	w m s	
	CCL2	0 (very small sample)	0	0 (very small sample)	0	scant epithelial cells
	ERBB2	2	w m s	2	w m	
	KRASV12 1 (one piece)	0		0		
	KRASV12 2 (2 other pieces)	1	m s	2	w m	
	MEKDD	1	w	4	w m s	
	PIM1	<5%	w (rare strong, 3- 4 cells)	2	m s	
RAF1	1	w m	4	w m	two pieces show this staining pattern; third piece has weak cyto stain in <25%, and nuclear stain weak in <25%.	
Castrated mice	NEK6 castr	most are neg; however, one tissue fragment shows up to 3+ Nuc score in ~25% of nuclei	0-3			

^aAR % score: 0, 1(1-25%),2(25-50%), 3(50-75%), 4(75-100%)

^bIntensity: w=Weak, m=Moderate, s=Strong (**bold** indicates predominant intensity)

Supplemental Table 2.

Gene Name	Site	q enriched (wt vs. kd)	q enriched (wt induced vs. uninduced)	modifiedsequence_localization
LIMCH1	S303	9.73E-64	1.72E-102	_SWSTATS(ph)PLGGERPFR_7
DROSHA	S357	7.81E-32	2.87E-68	_NTDSWAPPLEIVNHRS(ph)PS(ph)REK_18
FOXJ2	S8	4.12E-18	2.22E-06	_(ac)ASDLESS(ph)LTSIDWLPQLTLR_7
FOXO3	S7	1.06E-17	4.06E-54	_(ac)AEAPAS(ph)PAPLSPLEVELDPEFEPQSRPR_6
HUWE1	S2595	7.84E-13	1.09E-09	_LLGPSAAADILQLSSS(ph)LPLQSR_16
EPPK1	S1529	4.20E-12	2.03E-06	_QVS(ph)ARDLFR_3
COIL	S487	6.79E-12	1.13E-13	_KIDS(ph)PPIRR_4
SRGAP1	S932	6.79E-12	1.21E-25	_LLELTS(ph)SYSPDVSDYKEGR_7
SLC2A12	S244	6.95E-11	2.89E-05	_LRALS(ph)DTTEELTVIK_5
TRPS1	S843	2.12E-09	3.99E-13	_TLRDS(ph)PNVEAAHLARPIYGLAVETK_5
MTX1	S9	4.78E-06	3.80E-03	_(ac)MLLGGPPRS(ph)PR_9
OGFR	S349	9.83E-06	9.02E-07	_S(ph)VEPQDAGPLER_1
INTS3	S502	5.00E-05	2.61E-06	_FPEFCSSPS(ph)PPVEVK_9
TRA2B	S239	7.67E-05	3.27E-03	_S(ph)YRGGGGGGGWR_1
LMO7	S926	9.02E-05	5.17E-02	_GISS(ph)LPR_4
SATB2	S20	9.20E-05	5.42E-02	_SGS(ph)PDVKGPPPVK_3
ATM	T1885	1.32E-04	1.83E-02	_STT(ph)PANLDSESEHFFR_3
PLEKHA6	S313	3.42E-04	2.86E-06	_KSS(ph)MNQLQQWVNLRR_3
LMO7	S895	3.62E-04	2.13E-01	_VSAS(ph)LPR_4
PAK6	S246	4.80E-04	8.11E-07	_HGSEEARPQSCLVGSATGRPGGEGS(ph)PS(ph)PK_25
HNRNPM	S633	6.05E-04	3.70E-04	_GNFGGS(ph)FAGSFGGAGGHAPGVAR_6
MLLT3	S302	2.20E-03	9.42E-02	_KKS(ph)SSEALFK_3
HNRNPA2B1	S324	2.41E-03	5.43E-03	_SGNFGGS(ph)RNMGGPYGGNYGPGGSGGGYGGGR_7
NCOA5	S96	2.52E-03	1.18E-01	_DLRDS(ph)RDFR_5
SETX	T2474	2.53E-03	1.92E-01	_SLT(ph)HPPTIAPEGSRPQGGLPSSKLDGFAK_3
BCL6	S466	2.65E-03	2.40E-01	_SSSESHS(ph)PLYMHPPK_7
ATXN1	S811	2.94E-03	1.27E-03	_ICIEGRS(ph)NVGK_7
CDKN2AIP	S151	3.23E-03	1.47E-01	_VIEGKNS(ph)SAVEQDHAK_7
FAM21C	S288	3.49E-03	1.82E-01	_S(ph)RPTS(ph)FADELAAR_5
EPS8L1	T305	5.41E-03	3.80E-02	_AAGEGLLT(ph)LR_8
FOXA1	S307	5.42E-03	1.00E-01	_KDPSGASNPSADS(ph)PLHR_13
KLF4	T316	6.40E-03	6.99E-05	_TT(ph)PTLGLEEVLSR_2
LMO7	S1593	6.56E-03	1.32E-02	_SHS(ph)PSASQSGSQLR_3
ZNF326	S131	8.20E-03	1.57E-01	_NQGGSS(ph)WEAPYSR_6
PLEKHG6	S645	2.05E-02	1.29E-02	_S(ph)APELPEGILK_1
RIPK3	S316	3.04E-02	1.70E-03	_RFS(ph)IPESGQGTEMDGFRR_3
ZNF326	S106	3.98E-02	9.73E-09	_FGGS(ph)YGGRFESSYR_4
ERCC5	S156	4.94E-02	1.03E-01	_ENDLYVLPPLQEEKHHS(ph)S(ph)EEDEKEWQER_17
ERCC5	S157	4.94E-02	1.03E-01	_ENDLYVLPPLQEEKHHS(ph)S(ph)EEDEKEWQER_18
LIG1	S66	5.02E-02	9.97E-02	_VLGS(ph)EGEEDEALS(ph)PAK_4
MYOF	S193	5.29E-02	9.46E-04	_RMLS(ph)NKPQDFQIR_4
ZDHC18	S19	6.19E-02	1.13E-07	_(ac)MKDCEYQQISPGAALPAS(ph)PGAR_19
PBRM1	S353	6.53E-02	1.45E-09	_LSAITM(ox)ALQYGS(ph)ES(ph)EEDAALAAAR_12

EXPH5	S1444	7.11E-02	3.82E-02	_RSS(ph)WECTGSGR_3
SIPA1L3	S158	7.11E-02	5.33E-07	_SKDVEFQDGWPRS(ph)PGR_13
ATXN1	S238	7.85E-02	9.85E-02	_APGLITPGS(ph)PPPAQQNQYVHIS(ph)SSPQNTGR_9
KRT18	S323	8.16E-02	1.44E-01	_NLKASLENS(ph)LREVEAR_9
IRF2BP1	S453	9.61E-02	4.17E-02	_NVAEALGHSPKDPGGGGPVRAGGAS(ph)PAASSTAQPPTQHR_26
DLG3	Y673	1.01E-01	1.62E-01	_RDNEVDGQDY(ph)HFVVS_10
ATXN1	S775	1.02E-01	7.46E-03	_WS(ph)APESR_2
EXPH5	S341	1.12E-01	3.01E-02	_S(ph)LHFPATTQSK_1
RFX2	S28	1.27E-01	2.98E-02	_(ac)MQNSEGGADSPASVALRPSAAAPPVPAS(ph)PQR_28
PCYT1B	S315	1.59E-01	3.42E-02	_M(ox)LQALS(ph)PK_6
EPS8L1	T202	1.61E-01	2.35E-01	_AVIST(ph)VER_5
HIVEP2	S2300	1.81E-01	3.82E-02	_RGPHALQSSGPPSTPS(ph)SPR_17
ZNF608	S964	1.96E-01	4.92E-05	_SKASS(ph)PSDISSKDSVVK_5
KLF3	S71	2.08E-01	2.39E-23	_S(ph)SPPSAGNSPSSLKFPSSHRR_2
ACLY	S481	2.24E-01	2.45E-04	_KAKPAMPQDSVPS(ph)PR_13
ZNF608	S1453	2.31E-01	4.83E-07	_DRHS(ph)PFGQR_4

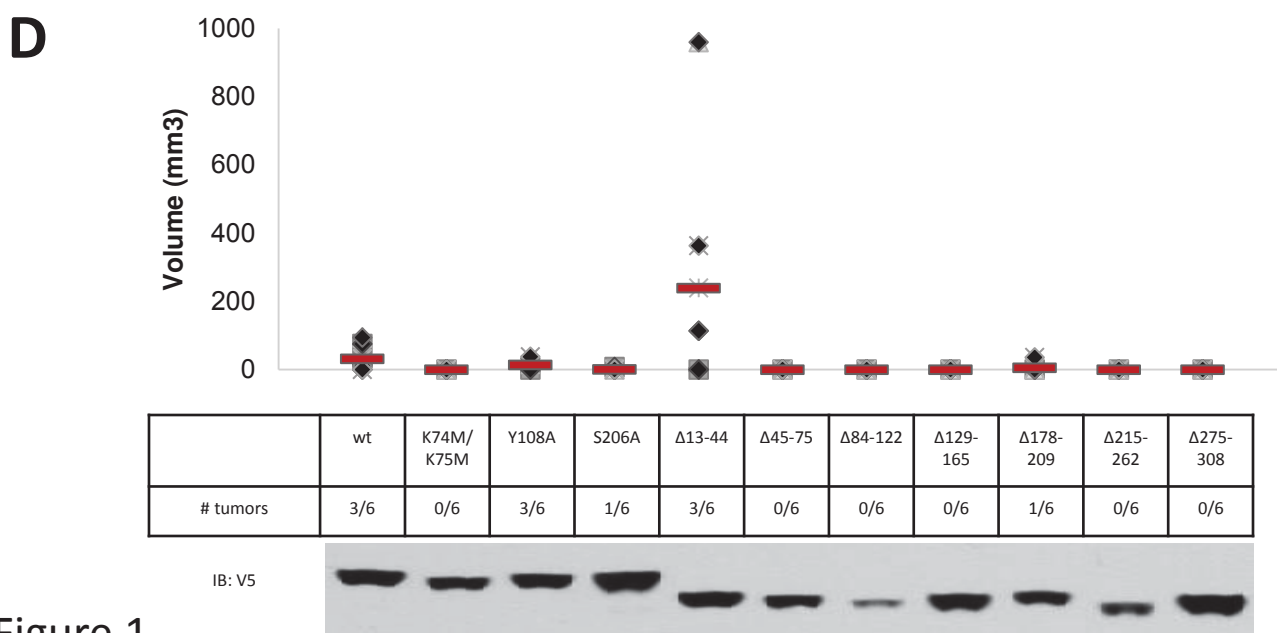
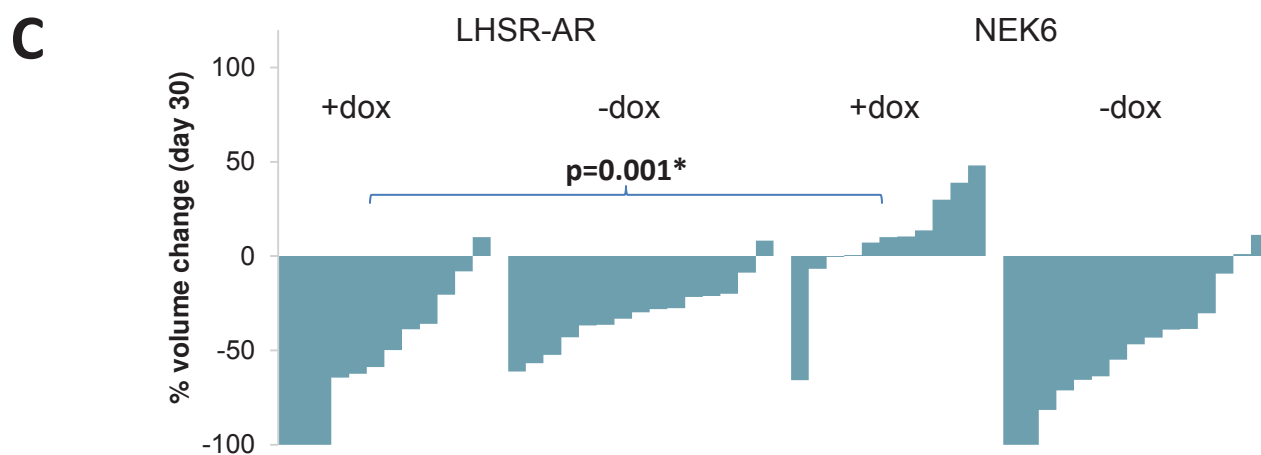
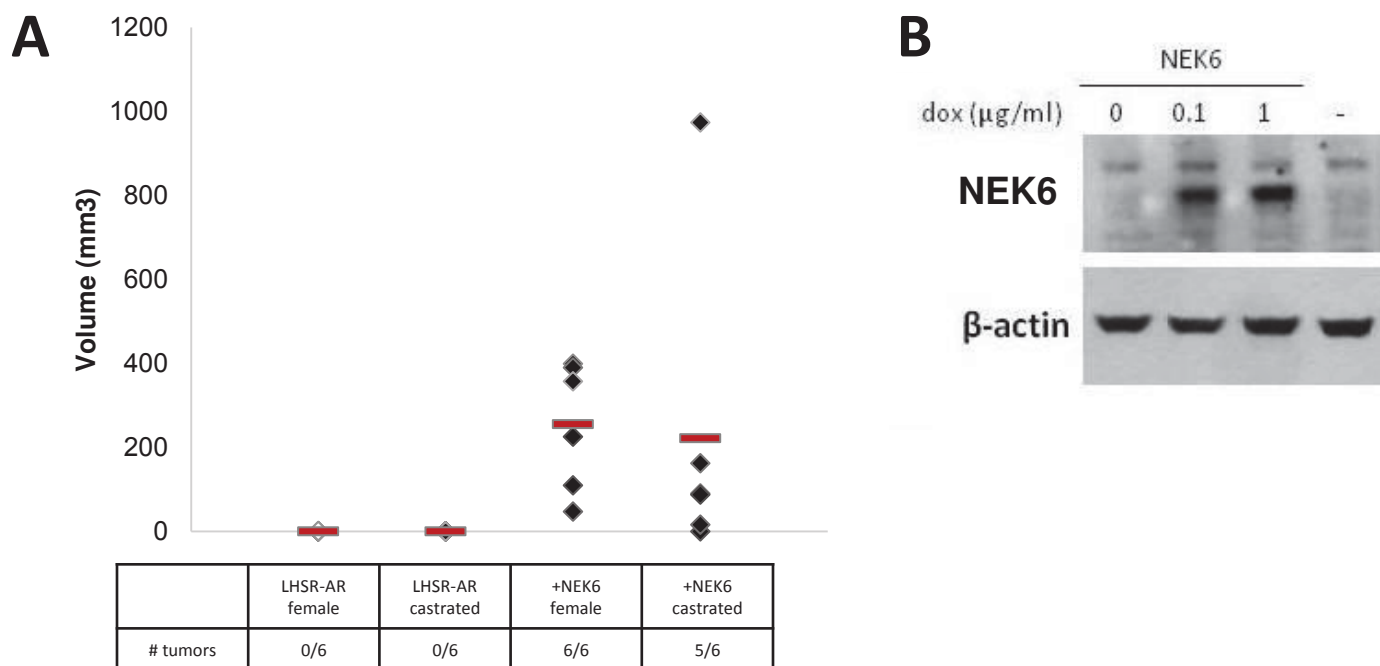


Figure 1

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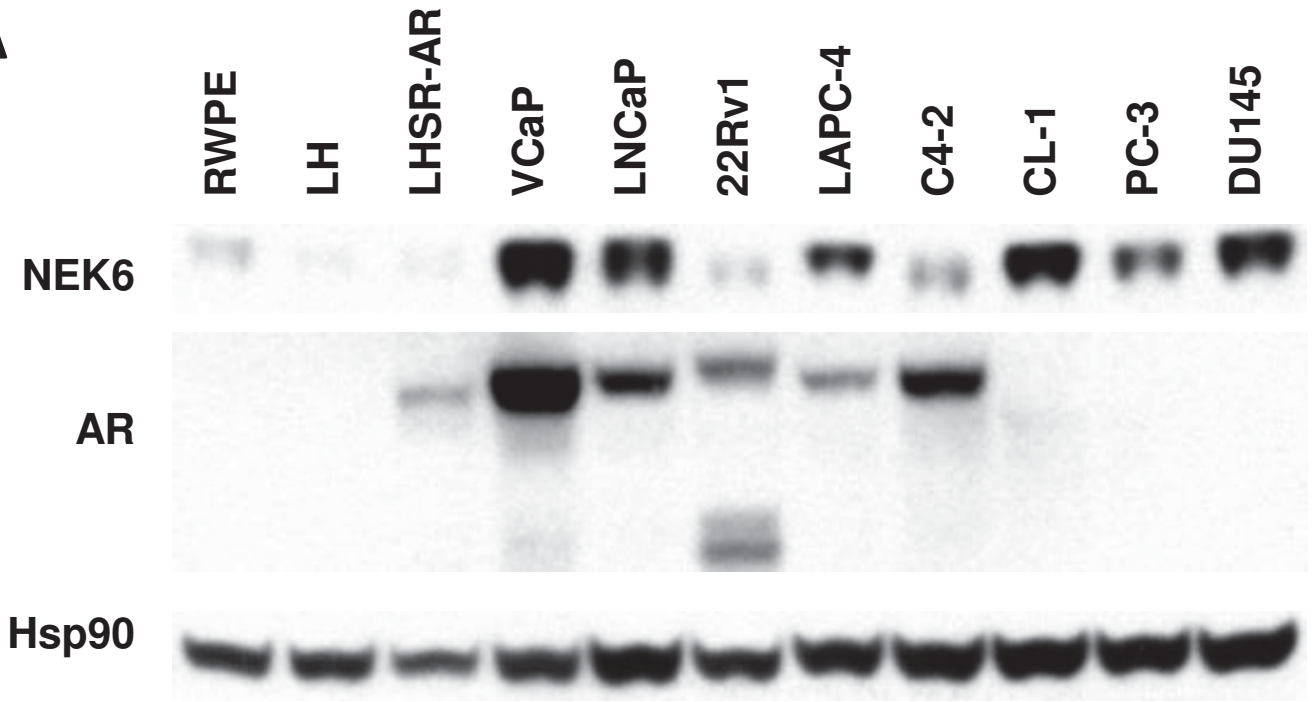


Figure 2

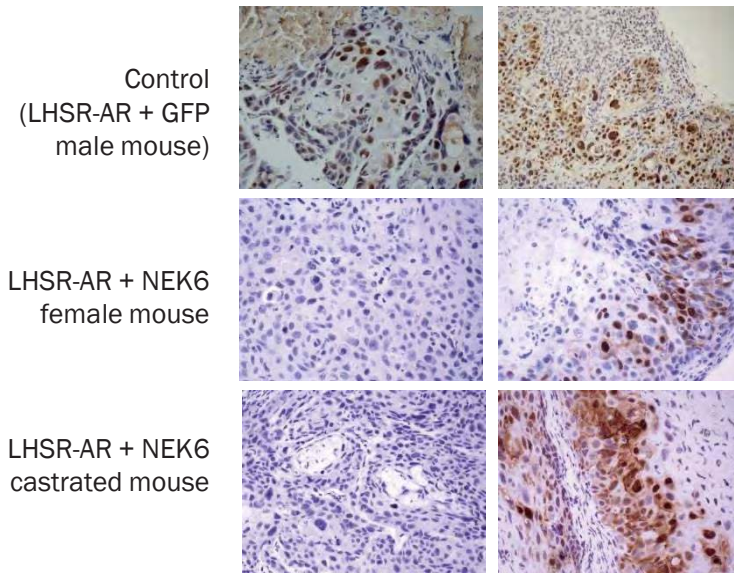
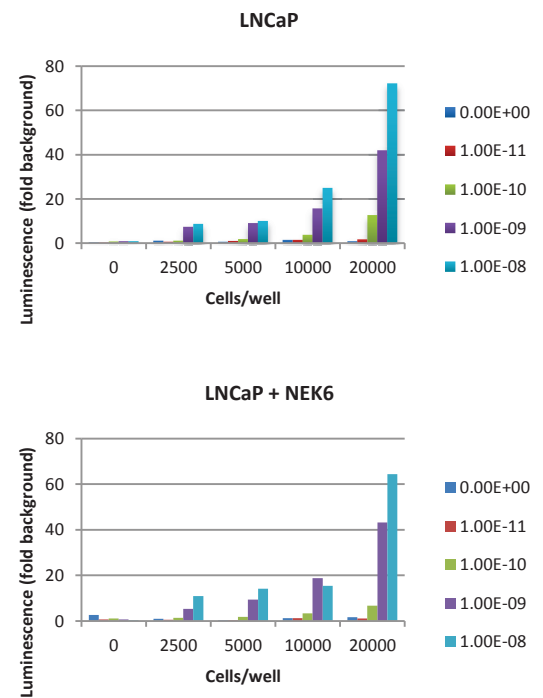
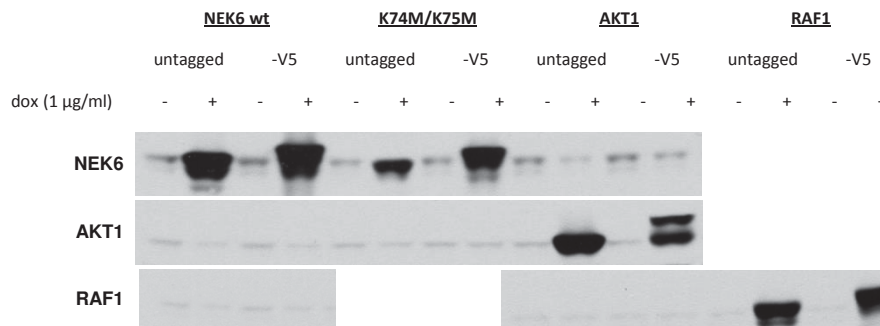
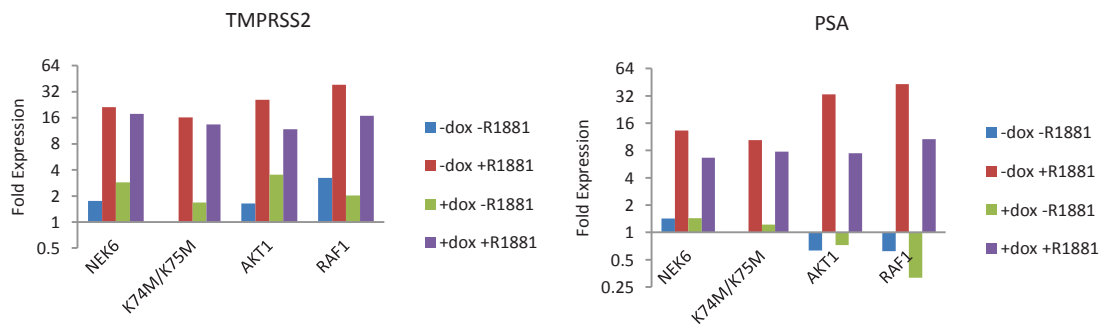
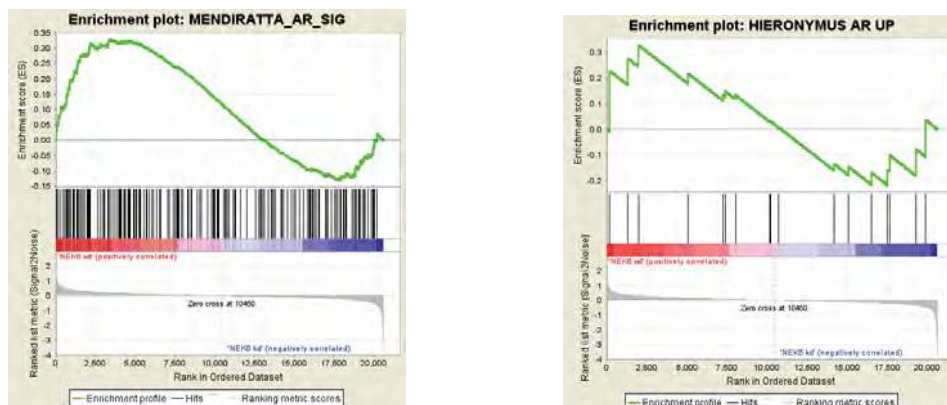
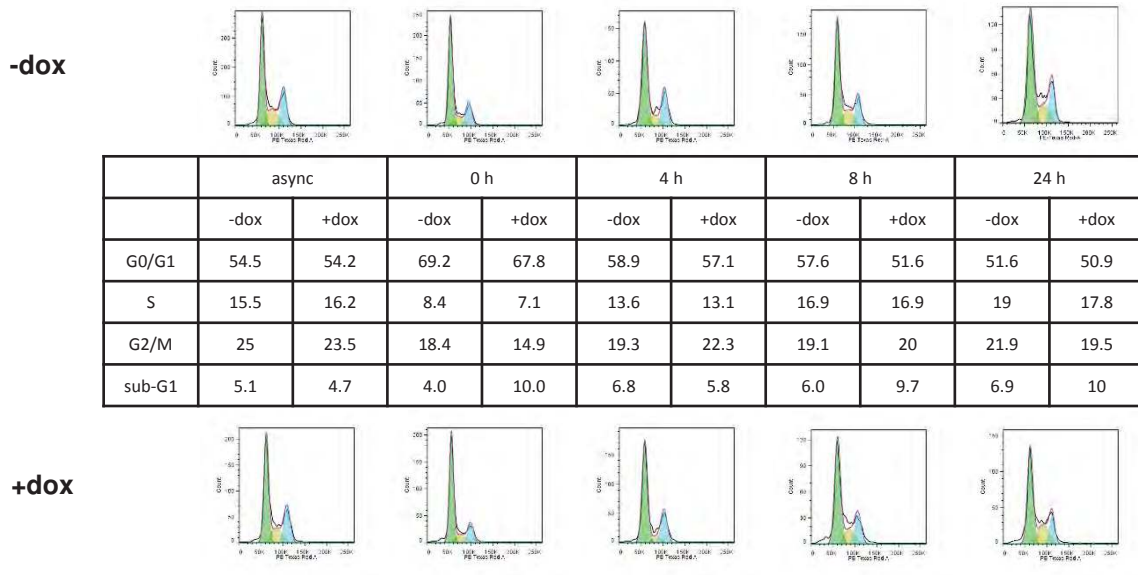
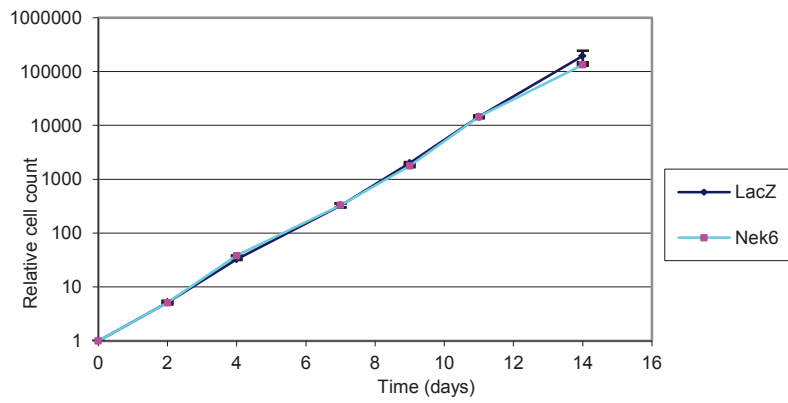
A**B****C****D****E**

Figure 3

A



B



C

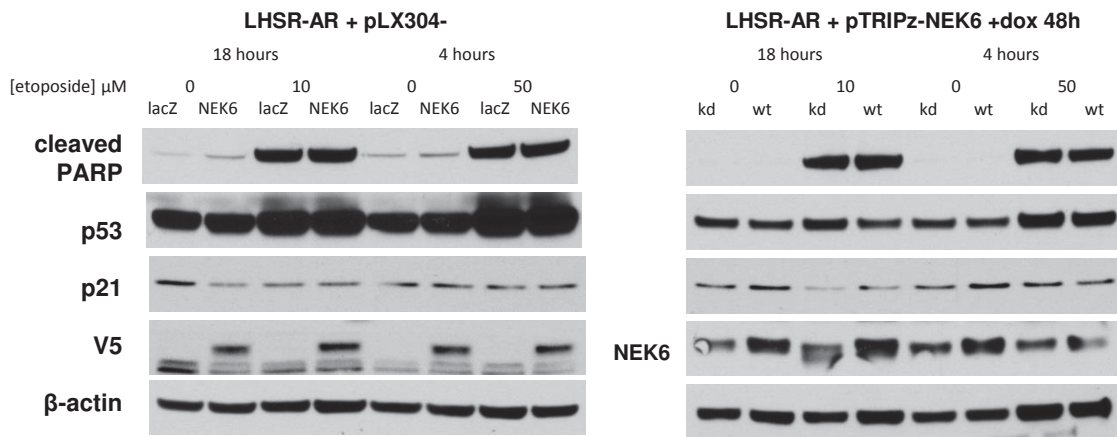


Figure 4

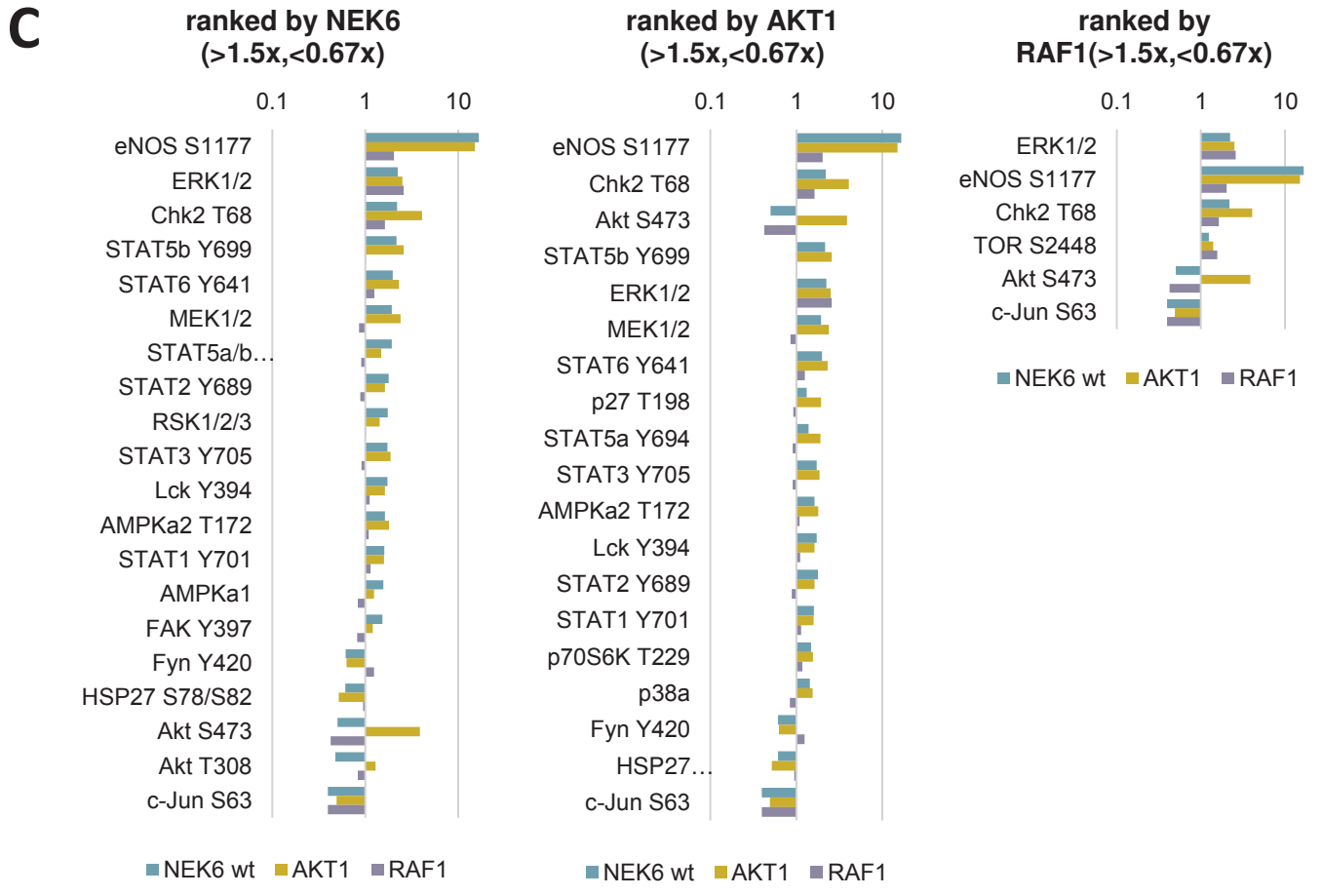
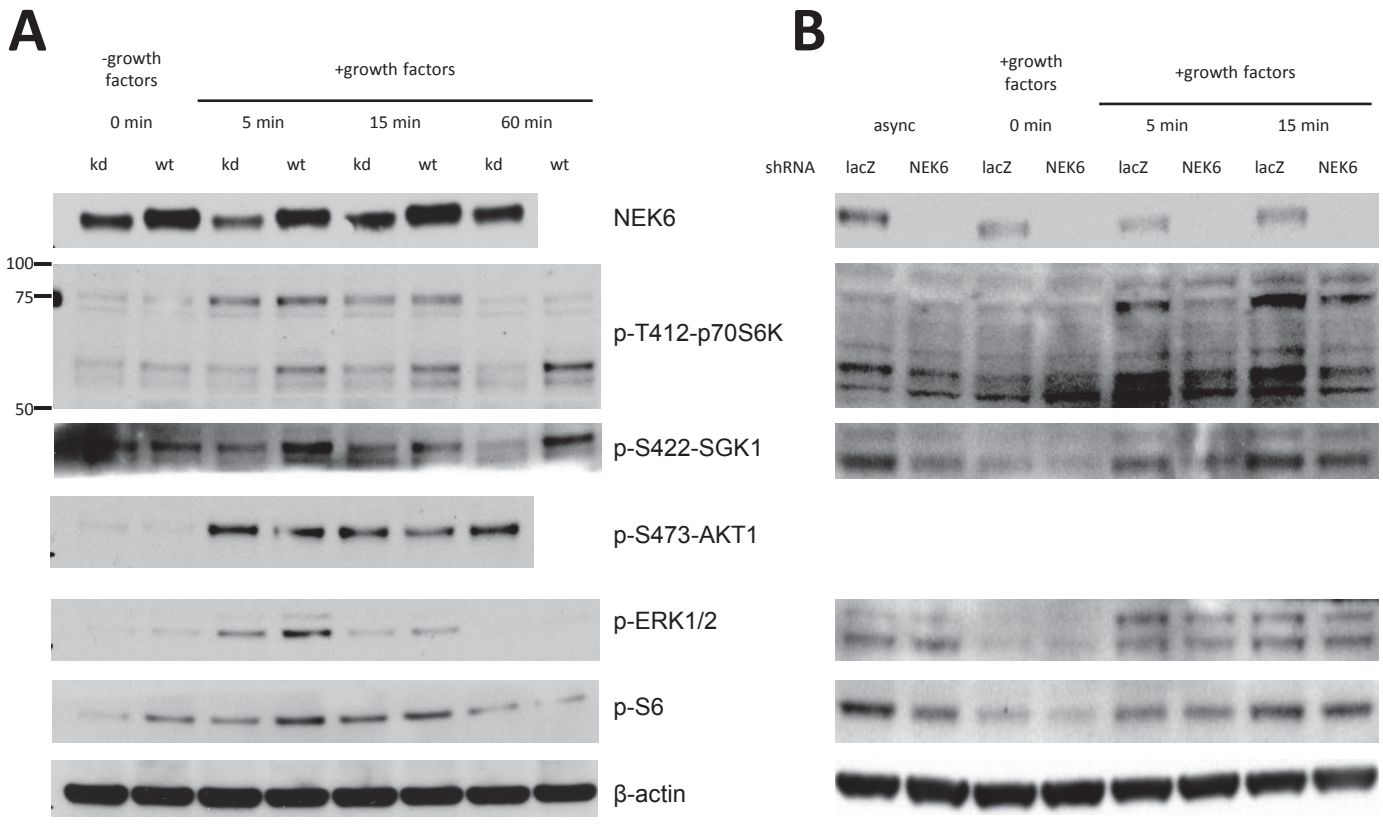


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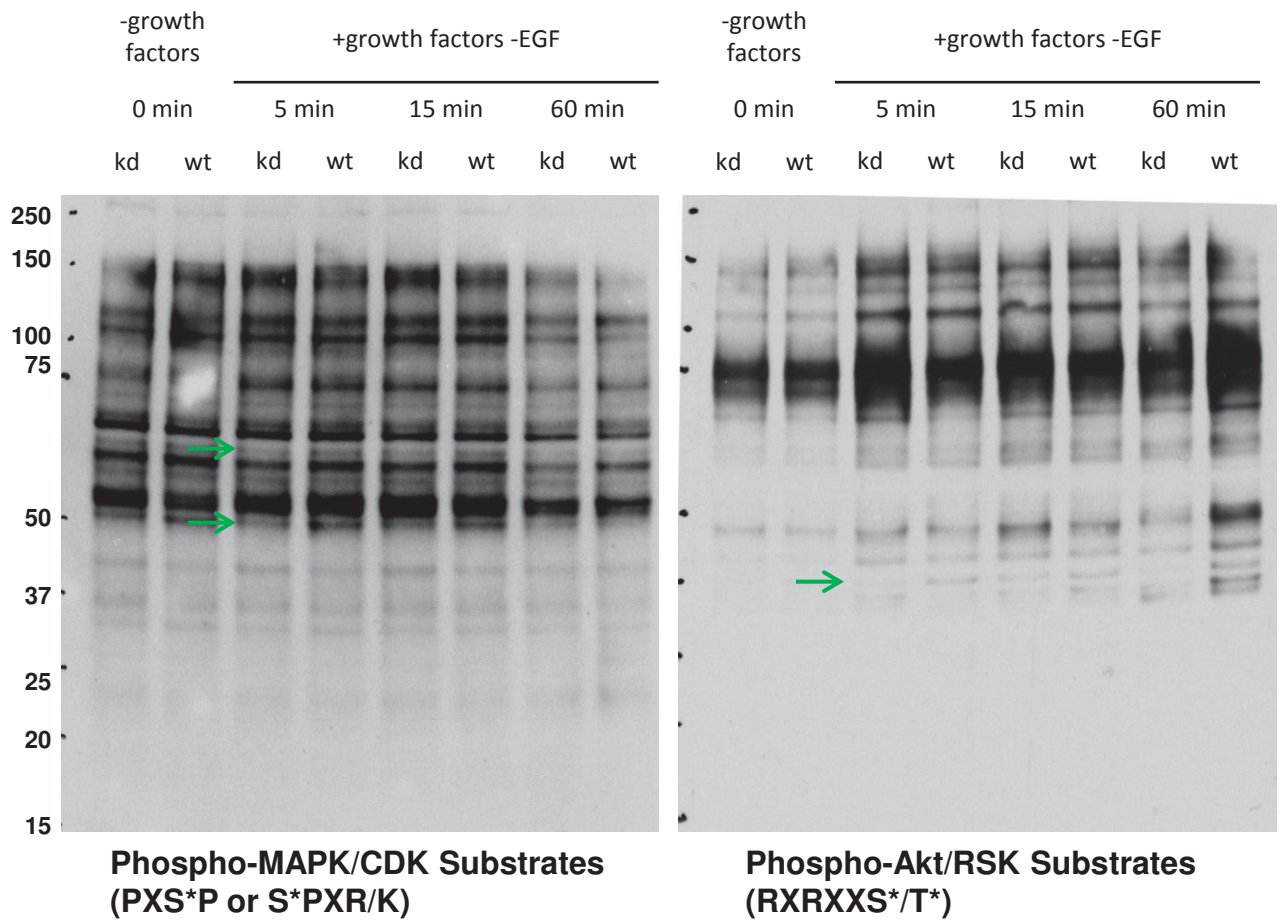
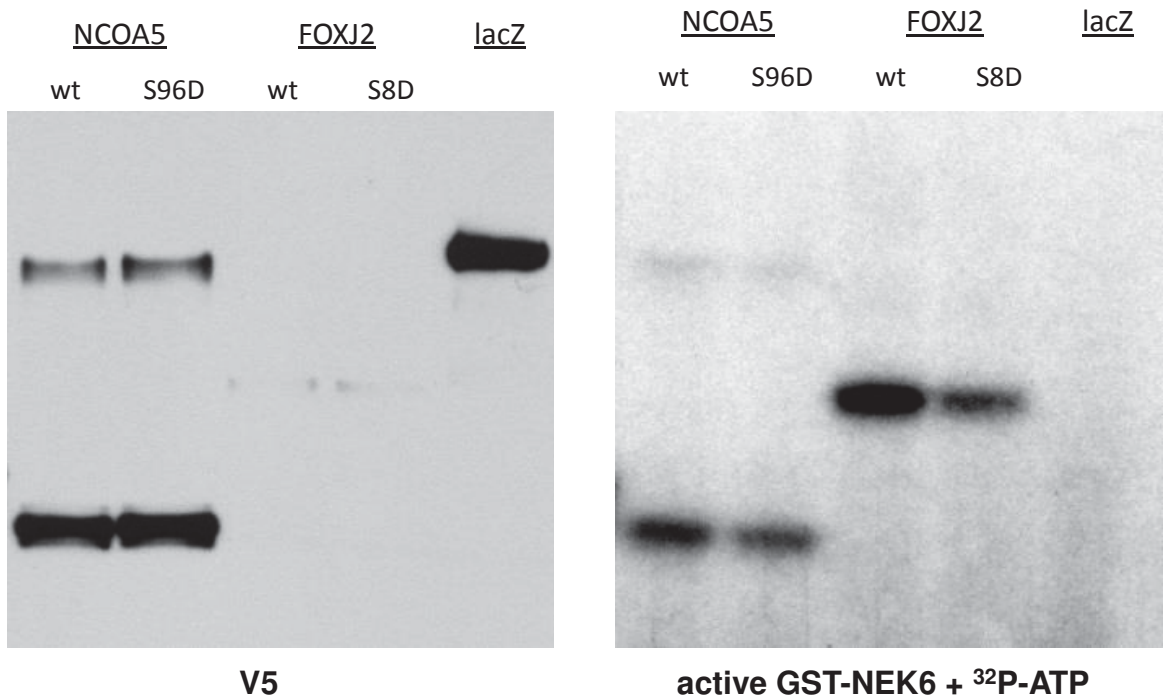
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Figure 6

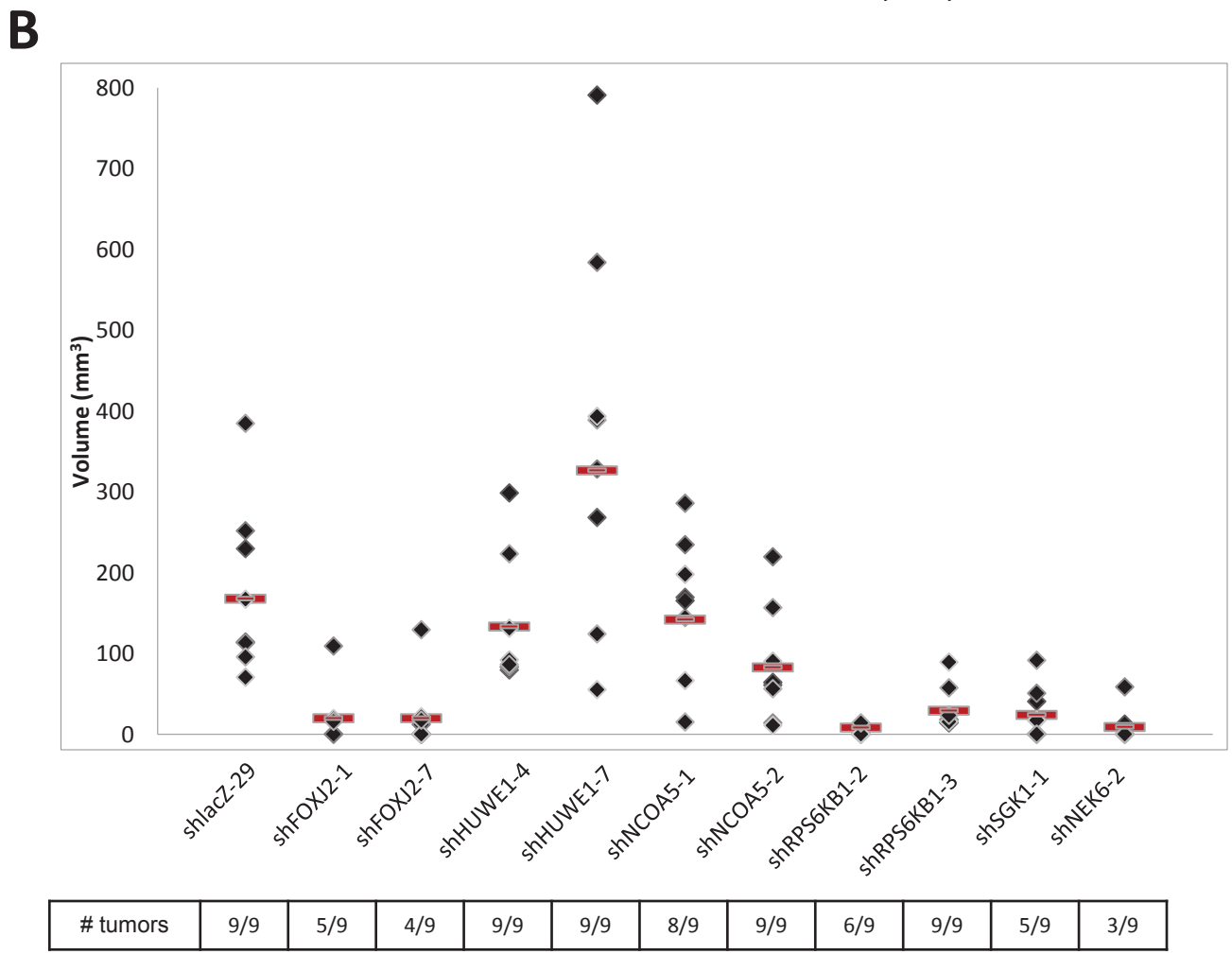
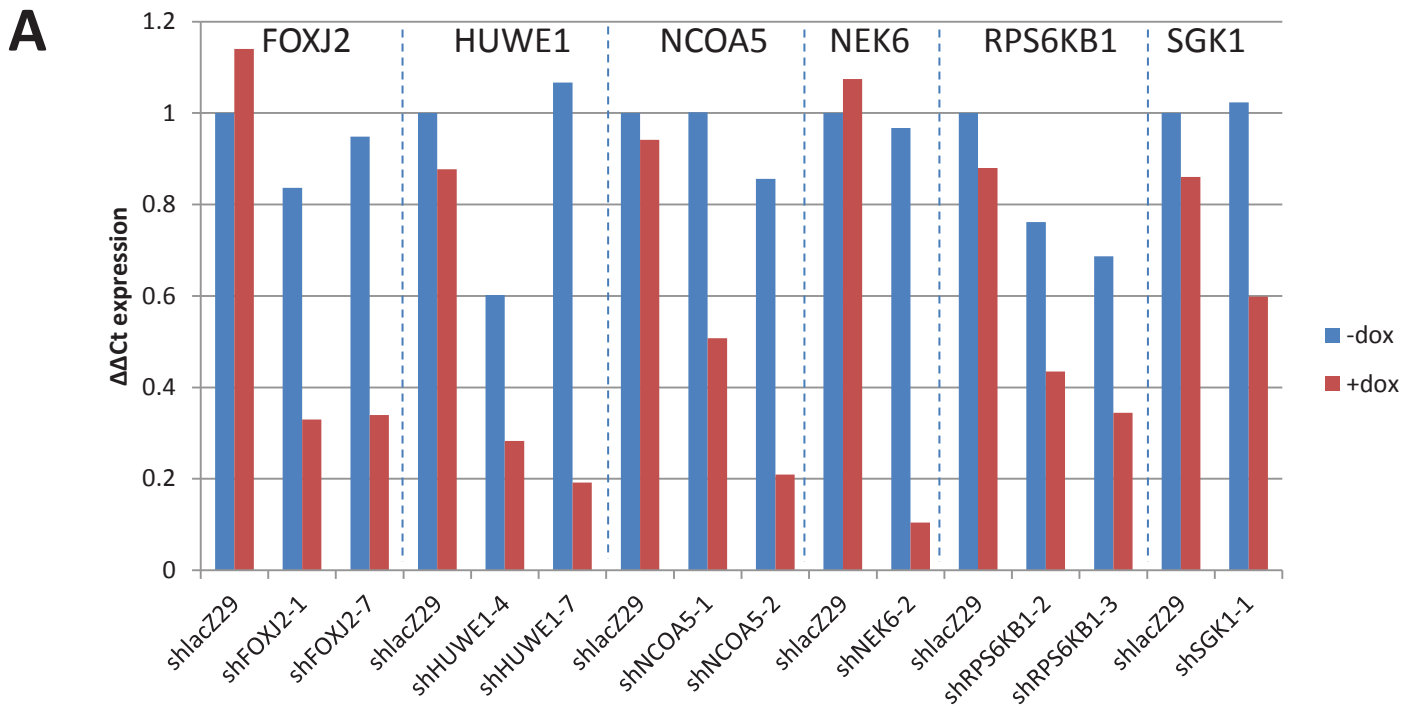


Figure 7

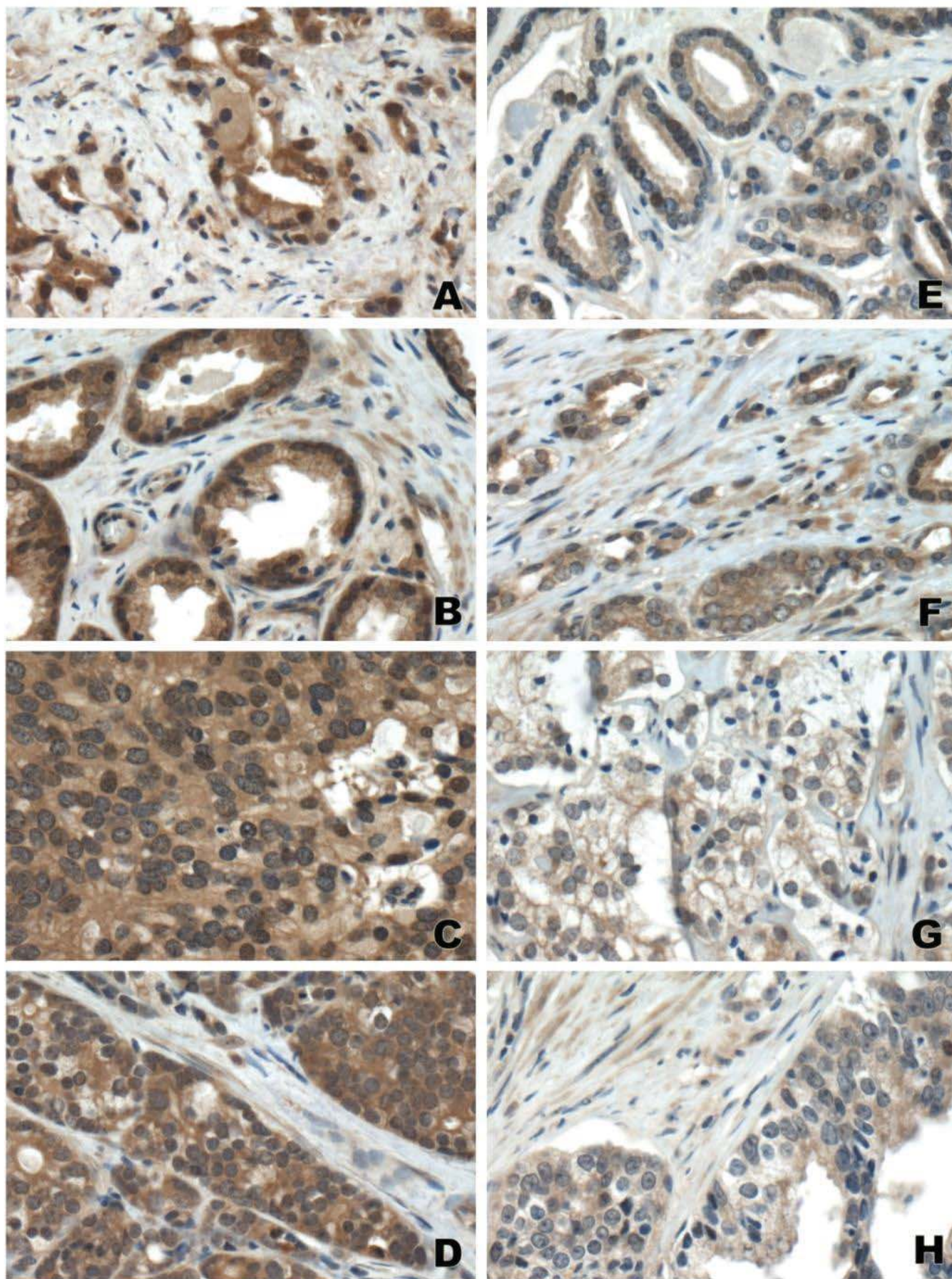


Figure 8

A

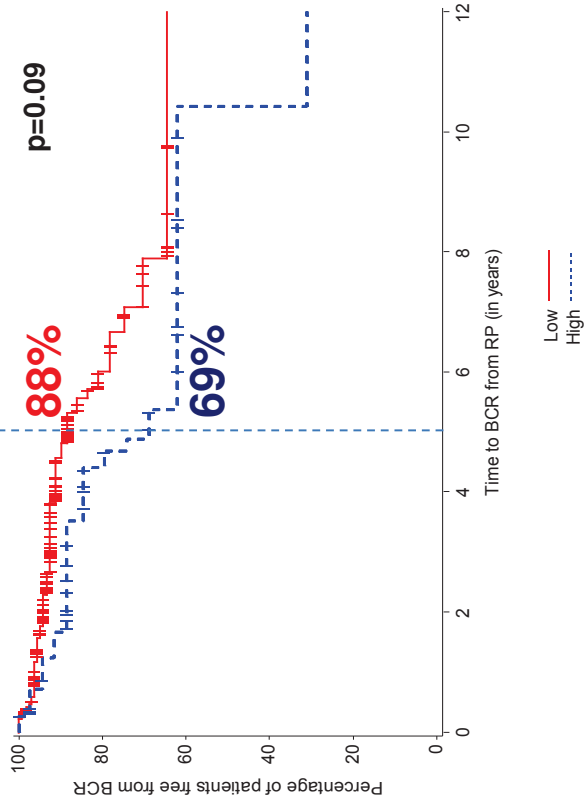
Gleason	NEK6 high	NEK6 low
≥8	3	9
7	21	78
≤6	17	87
$\chi^2 = 1.07$ (p=0.5857)		

	NEK6 high	NEK6 low
Recurrent	10	20
Non-recurrent	30	148
χ^2 (Yates') = 3.49 (p=0.062)		

	NEK6 high	NEK6 low
Metastatic	5	7
Non-metastatic	35	161
χ^2 (Yates') = 2.74 (p=0.098)		

	NEK6 high	NEK6 low
Hormone refractory	7	7
Other	33	161
χ^2 (Yates') = 7.15 (p=0.0075)		

B



C

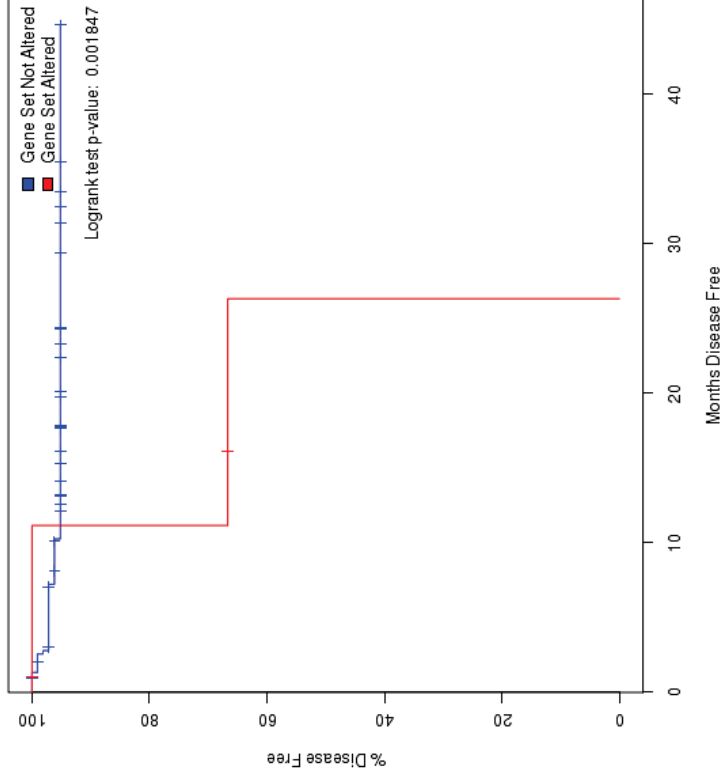


Figure 9