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Kinase and Hormone Receptor Signaling Networks in Cancer

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Kinase and Hormone Receptor Signaling Networks in Cancer

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular & Medical Pharmacology

By

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ABSTRACT OF THE DISSERTATION

Kinase and Hormone Receptor Signaling Networks in Cancer

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Doctor of Philosophy in Molecular & Medical Pharmacology

University of California, Los Angeles, 2012

Professor Charles Sawyers, Chair

Cell signaling networks govern basic cellular activities including cell growth, proliferation, survival and death. Dysregulation of signaling pathways caused by dysfunctional kinases and hormone receptors is commonly seen in human cancers. Ribosomal protein S6 (rpS6) has been indicated to be involved in determining cell size, cell proliferation and glucose homeostasis. The dissertation work dissected the regulation of S6 phosphorylation (pS6) by growth factor-driven pathways and amino acids in details. It challenged the common notion that pS6 is a good readout for the PI3K/Akt pathway in therapeutic evaluation by showing that pS6 received inputs from and could potentially depend on both overactivated PI3K and MAPK pathways in cancer cells. Furthermore, it provided evidence that a sustained amino acid input to mTORC1 was sufficient to cause pS6 and cell proliferation resistance to Akt and MEK inhibitors, which raised the possibility to target the amino acids/mTORC1 axis in anticancer treatment. The dissertation also

comprised a functional study of constitutively active androgen receptor (AR) splice variants in castration resistant prostate cancer (CRPC). By using immunoflurescent staining, luciferase reporter assay and xenograft animal models, we showed that only constitutively nuclear localized AR variants displayed transcriptional activity and growth advantage. However, those variants lost their activity when full length AR was blocked either pharmacologically or genetically, hence were not considered the cause of drug resistance in CRPC.

The dissertation of Yinan Chen is approved.

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To my loving parents, Chen Jie and Chen Wei, who have been supporting me with their unconditional love and encouragement. You are my role models and lifelong inspiration. I hope I have made you proud.

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Vita

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Publications

A. Journals

1. *Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor* Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, Kim K, Sawyers CL Proc Natl Acad Sci U S A. 2010 Sep 7.

2. *Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma* Cloughesy TF, Yoshimoto K, Nghiemphu P, Brown K, Dang J, Zhu S, Hsueh T, Chen Y, Wang W, Youngkin D, Liau L, Martin N, Becker D, Bergsneider M, Lai A, Green R, Oglesby T, Koleto M, Trent J, Horvath S, Mischel PS, Mellinghoff IK, Sawyers CL PLoS Med. 2008 Jan 22;5(1):e8.

B. Meetings and conferences

1. *mTORC2-independent modulation of the negative feedback loop in PI3-kinase/Akt pa*thway Yinan-Fiona Chen, Igor Vivanco, Ingo K. Mellinghoff, Charles L. Sawyers Phosphorylation, Signaling & Disease May 16-May 20, 2007 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: Academic press 26

Chapter 1

Introduction to the ribosome protein S6

S6 ribosomal protein is a component of the 40S ribosomal subunit

Ribosomes are found in all living cells and where translation takes place. In eukaryotic cells, ribosomes are composed of 40S (small) and 60S (large) units. The small unit binds messenger RNA (mRNA) while the large unit binds transfer RNA (tRNA) and amino acids [1, 2]. mRNA is transcribed from DNA and serves as a template by which the correct sequence of amino acids in a protein is determined. During translation, tRNAs brings amino acids to the ribosome according to the mRNA encoded codons, and the assembled amino acids are then joined together as the ribosome moves along the mRNA molecule (Fig 1-1) [3]

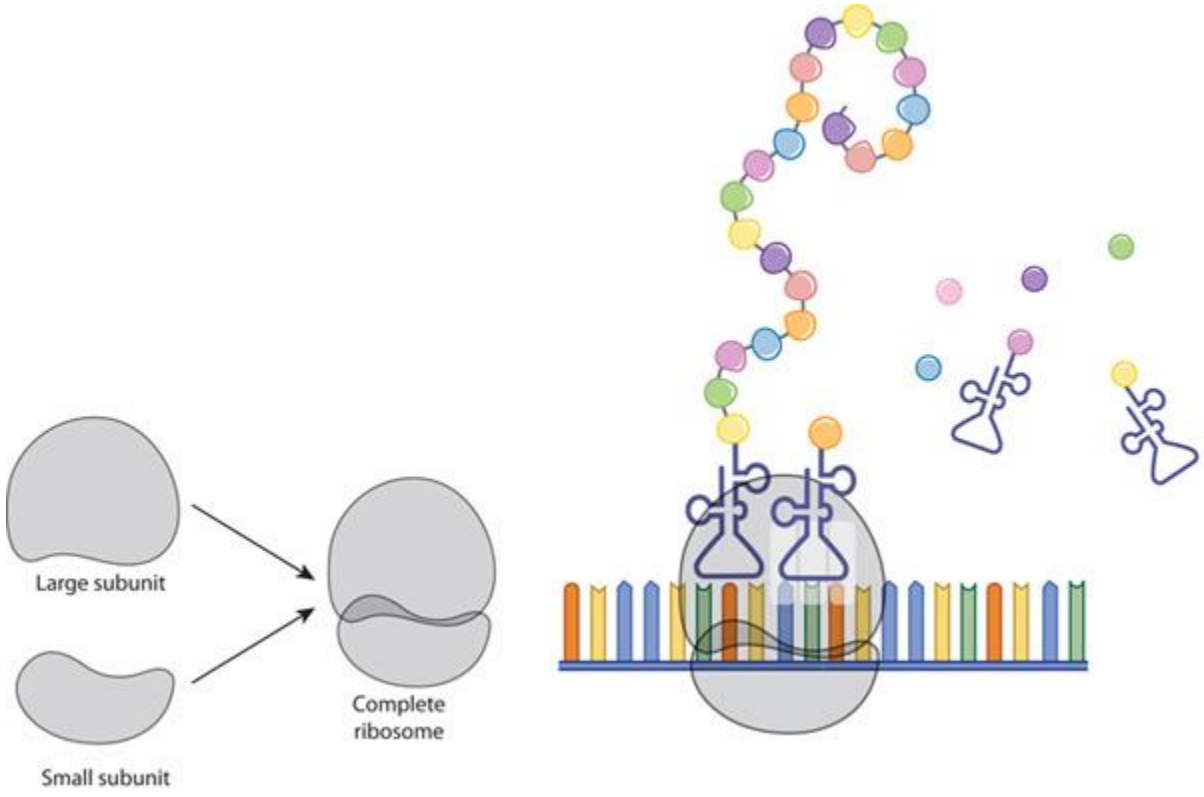

Figure 1-1 The ribosome and translation© 2010 Nature Education

The ribosome subunits are composed of ribosomal RNA (rRNA) and ribosomal proteins. The small subunit contains an 18S rRNA and 33 proteins, including ribosomal protein S6 (rpS6). Recent progress in crystallization revealed the detailed structure of rpS6 as well as its position in the 40S subunit (Fig 1-2) [4].

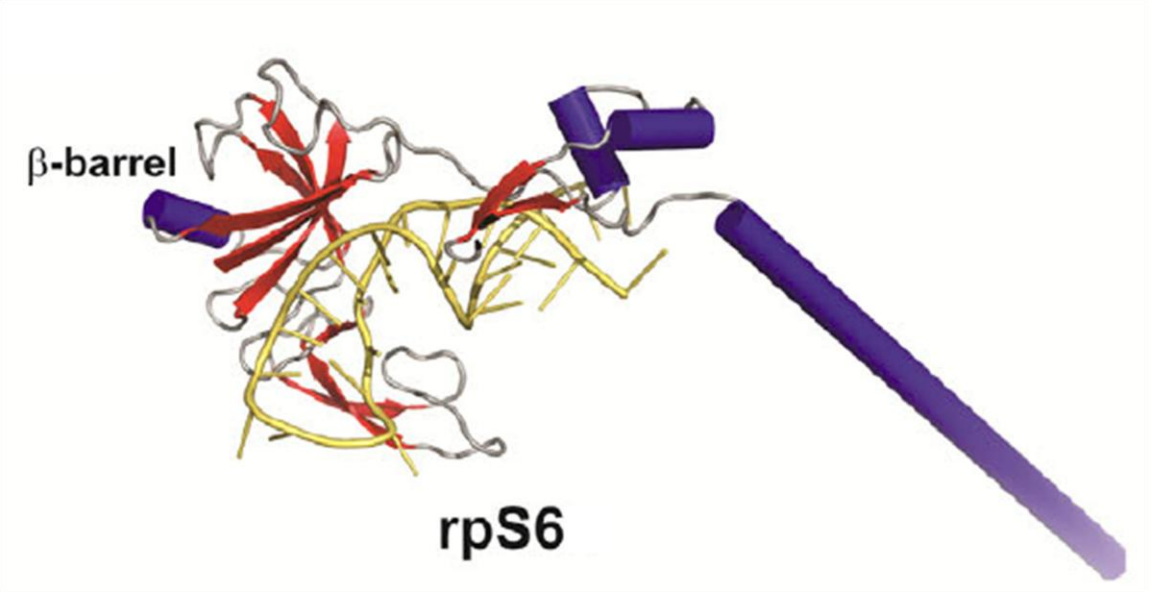

Fig 1-2A Fold of rpS6 showing the N-terminus β-barrel domain and the long C-terminus α-helix domain.

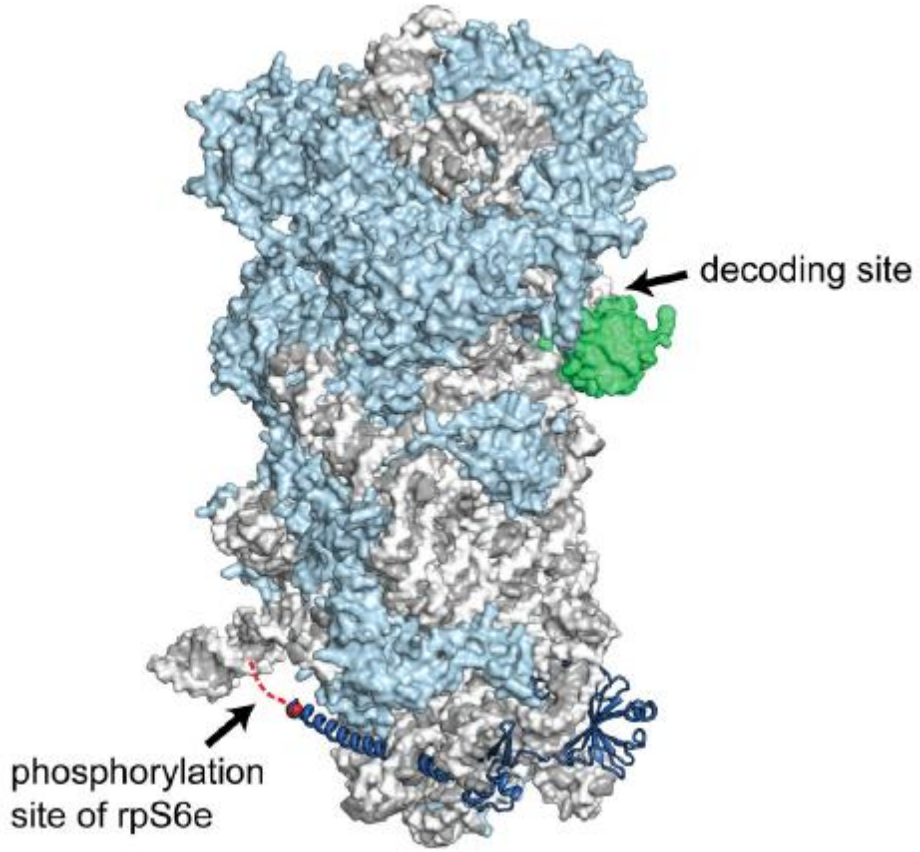

Fig 1-2B Position of rpS6 in the ribosome 40S unit. The 40S subunit is shown in side view with the rRNA in gray surface and proteins in light blue surface. rpS6 is shown as blue cartoon with phosphorylation sites indicated by a red dash line. Eukaryotic initiation factor 1 (eIF1) is shown in green.

Phosphorylation sites of rpS6

In 1970, David Kabat discovered a 33KD protein that is associated with the small subunit of ribosome undergoes phosphorylation in rabbit reticulocytes [5]. This protein was referred to as "protein F" and later identified as rpS6 [6]. Subsequent studies map the phosphorylation sites of rpS6 in mammals to five serine residuals: S^{235}, S^{236}, S^{240}, S^{244}, and S^{247} [7, 8]. All five residuals

reside in the C-terminus of rpS6 (see Fig 1-2A) and are evolutionarily conserved (Table 1-1) [7-10].

Table 1-1 Sequences of the C-terminus of rpS6 from different eukaryotes. Red letters denote phosphorylation sites; blue letters denote putative phosphorylation sites on the basis of complete sequence conservation among the vertebrate homologues of rpS6 [11].

Saccharomyces cerevisiae (bakers yeast)	AEKAEIRKRRASSLKA^{236}
Drosophila melanogaster (fruit fly)	RRRSASIRESKSSVSSDKK^{248}
Oncorhynchus mykiss (rainbow trout)	RRRLSSLRASTSKSESSQK^{249}
Xenopus laevis (Afircan clawed frog)	RRRSSLRASTSKSESSQK^{249}
Gallus gallus (chicken)	RRRLSSLRASTSKSESSQK^{249}
Rattus norvegicus (rat)	RRRLSSLRASTSKSESSQK^{249}
Mus musculus (mouse)	RRRSSLRASTSKSESSQK^{249}
Canis familiaris (dog)	RRRLSSLRASTSKSESSQK^{249}
Homo sapiens (human)	RRRLSSLRASTSKSESSQK^{249}

rpS6 is phosphorylated by multiple kinases

The first rpS6 kinase was identified as a 90KD protein in *Xenopus* oocytes after mitogenic stimulation, and termed as p90 ribosome protein kinase (RSK) [12]. A 65-70KD protein is found to be the dominant kinase phosphorylating rpS6 in avian and mammalian cells and referred to as

S6K [13, 14]. Subsequent studies found two forms of S6K in mammalian cells, S6K1 and S6K2, which are encoded by two different genes but share a high level of sequence homolog [15-17].

S6K1 and 2 function at least partially redundantly based on the evidence that phosphorylation of rpS6 proceeds normally upon mitogen stimulation in S6K1-/- and S6K2-/- mouse embryonic fibroblasts (MEFs) [18, 19]. Both forms are required for rpS6 phosphorylation since only when both S6K1 and S6K2 are existent is full rpS6 phosphorylation seen. Notably, in S6K1(-/-)/S6K2(-/-) double knockout cells, rpS6 phosphorylation at Serine 235 and 236 is still detected, probably by RSK.

RSK is downstream of the extracellular signal-regulated kinase (ERK) and plays a key role in regulating cell division, proliferation and survival [20]. There are four forms of RSK (RSK1-4) encoded by four different genes have been identified. Although S6K is considered to be the dominant kinase of rpS6 in somatic cells, the involvement of RSK is proved by the persistent Serine 235 and 236 phosphorylation in S6K1(-/-)/S6K2(-/-) cells, which is abolished by treatment by inhibitors to either mitogen-activated protein kinase (MAPK) or ERK, both of which are upstream activators of RSK [19]

Role of rpS6 phosphorylation

Some early studies using ultraviolet cross-linking experiments showed that rpS6 interacts with tRNA, translation initiation factors and mRNA, suggesting that phosphorylation of rpS6 plays a role in translation initiation [21]. The original widely accepted model is that 40S subunits

containing phosphorylated rpS6 had a selective advantage to be recruited to polysomes [22-24]. However, later studies showed that phosphorylation of rpS6 by itself is not sufficient to facilitate the mobilization of 40S subunit to polysomes [25-27]. The conflictive evidence of the role of rpS6 phosphorylation is not resolved until the generation of the rpS6 phosphorylation null knockin mouse model ($rpS6^{p-/-}$), in which all phosphorylatable serine residuals in rpS6 are replaced with alanine [28]. Using this model, researchers found that rpS6 phosphorylation is dispensable for the recruitment of liver ribosomes to polysomes. Furthermore, the mouse model showed that the translation efficiency of mRNA with a 5'-terminal oligopyrimidine tract (referred to as TOP mRNA) is controlled in a rpS6 phosphorylation independent manner, despite the fact that it is correlated with the level of rpS6 phosphorylation level under many physiological conditions (reviewed in [29]). Consistently, crystal structure of ribosome 40S subunit revealed that the C-terminus phosphorylation sites of rpS6 is at least 130Å from the decoding center (see Fig 1-2B) [4].

It is noted that at least certain types of cells in the $rpS6^{p-/-}$ are smaller in size, indicating that rpS6 phosphorylation plays a role in determining cell size, consistent with the involvement of S6K signaling pathway [30-32]. However, the birth weight of $rpS6^{p-/-}$ mice is normal despite the smaller cell size, which is then found to be explained by faster cell proliferation rate. The inverse relationship between rpS6 phosphorylation and cell proliferation rate is further supported by cell cycle arrest caused by oncogenic Ras in *Xenopus* egg extracts with high level of phospho-rpS6 [33].

One of the cell types that are smaller in size in rpS6$^{p-/-}$ mouse is the pancreatic β-cells [28]. It has known before that insulin secretion is closely correlated with the size of β-cells [31, 34]. Not surprisingly, rpS6$^{p-/-}$ mouse displays higher and longer hyperglycemia after glucose challenge due to insufficient insulin secretion. The recapitulation of rpS6$^{p-/-}$ mouse to S6K1-/- mouse in insulin intolerance suggests that it is the phosphorylation of rpS6 that plays a key role in glucose homeostasis.

Regulation of rpS6 phosphorylation

rpS6 phosphorylation can be induced by many upstream stimuli and is under the regulation of a complicated signaling network. In this section of the dissertation, we will review the current knowledge of phospho-rpS6 regulation.

Stimuli that induce rpS6 phosphorylation

Since the identification of rpS6 in rabbit reticulocytes in 1970, this 33KD protein has been in the center of interest since it was the only ribosome protein that undergoes phosphorylation in rat liver regeneration, and for many years considered to be the only substrate of S6K. Subsequent studies have shown that rpS6 phosphorylation can be induced by multiple stimuli (Table 1-2).

Table 1-2 rpS6 phosphorylation is inducible by many stimuli (reviewed in [49])

Treatment	Organism/Cells	References
A. Mitogen		
a. Liver regeneration	Rat	[35]
b. Growth factors and cytokines		
(1) Serum, IGF	Chicken embryo fibroblasts	[36]
(2) EGF	Mouse Swiss 3T3 cells	[24]
(3) NGF	Rat PC12 cells	[37]
(4) PDGF	Mouse Swiss 3T3 cells	[38]
(5) Interleukin 2	Mouse T lymphocytes	[39]
B. Hormones		
a. Insulin	Mouse 3T3-L1 cells	[40]
b. Glucagon	Rat liver	[41]
c. Progesterone	*Xenopus* ooycyte	[42]
d. Estrogen	Rooster hepatocytes	[43]
e. PTTH	Tobacco hornworm	[44]

f. Juvenile hormone	Flesh-fly	[45]
C. Nutrients		
a. Amino acids	Human HEK293 cells	[46]
b. Leucine	Rat L6 myoblast	[47]
c. Glucose	Mouse MIN6 β-cells	[48]
D. Lipid compounds		
a. Diacyl glycerol	Mouse T lymphocytes	[39]
b. Prostaglandin $F_{2\alpha}$	Mouse Swiss 3T3 cells	[10]
E. Viral infection		
a. Vaccinia virus	Human Hela cells	[50]
b. Pseudorabies	Hamster fibroblasts	[51]
c. Simian virus	Hamster fibroblasts	[51]
d. Avian sarcoma virus	Chicken embryo fibroblasts	[52]
e. AMLV	Mouse NIH 3T3 fibroblasts	[53]
F. Pharmacological agents		
a. Translation inhibitors		

(1) Cycloheximide	Rat liver	[54]
(2) Puromycin	Rat liver	[54]
b. Transcription inhibitor		
(1) D-glactosamine	Rat liver	[55]
c. Phorbol ester	Rat hepatoma cells	[56]

IGF, insulin-like growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTTH, prothoracicotropic hormone; AMLV, Abelson murine leukemia virus.

Notably, phospho-rpS6 can also be downregulated by multiple factors (not listed, reviewed in [49]).

Pathways that regulate rpS6 phosphorylation

Many pathways have been found to be involved in the regulation of rpS6, including growth-factor driven PI3K/Akt and Ras/MEK/MAPK pathways, amino acids, energy balance, oxygen supply and osmolarity (summarized in [49]). In the current study, we dissected phospho-rpS6 regulation by growth-factor driven pathways and amino acids in details under physiological and oncogenic conditions. For more information on those pathways, see introduction in Chapter 2 and 3, respectively.

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Chapter 2

Regulation of S6 ribosome protein phosphorylation by PI3K and MAPK pathways

Introduction

PI3K/Akt/mTOR pathway

The phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway controls many important cellular processes in both physiological and pathological conditions, including transcription, translation, cell cycle progression and metabolism [1, 2]. Targeting various components of this pathway has been of great interest in anticancer research.

Growth factors such as insulin and insulin-like growth factors (IGFs) activate the PI3K pathway by binding receptor tyrosine kinases (RTKs), which leads to the recruitment of insulin receptor substrate (IRS) and the subsequent recruitment of PI3K. PI3K then converts phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3) in the cell membrane. The tumor suppressor gene phosphatase and tensin homolog deleted on Chromosome 10 (PTEN) antagonizes the accumulation of PIP3 by specifically dephosphorylating the 3' phosphate. Proteins with pleckstrin-homology (PH) -domain such as Akt and phosphoinositide-dependent kinase 1 (PDK1) are recruited to cell membrane by directly binding to PIP3, resulting in the phosphorylation and activation of Akt by PDK1 (reviewed in [1]). Rapamycin insensitive mTOR complex 2 (mTORC2) also phosphorylates Akt, but the detailed mechanism of mTORC2 activation is yet to be determined [3]. The link between Akt and rapamycin sensitive mTOR complex 1 (mTORC1) is the tuberous sclerosis proteins TSC1 and TSC2. TSC1 and 2 form a heterodimer and act as a GTPase-activating protein (GAP) for the GTPase Ras homolog enriched in brain (Rheb) [4]. Akt phosphorylates and inactivates TSC1/2

complex upon insulin stimulation, resulting in the activation of Rheb. Rheb directly binds to the kinase domain of mTOR and activates mTOR in a GTP-dependent manner [5].

MAPK pathway

The mitogen-activated protein kinase (MAPK) cascade comprised of Ras, Raf, MEK, and extracellular signal-regulated kinase (ERK) is another pathway that responds to growth factors and controls cell proliferation, survival, motility and metabolism. Like the PI3K pathway, overactivated Ras/ERK pathway is commonly seen in human cancers and has been widely studied in anticancer target therapy [6, 7].

Cell membrane-localized RTKs are activated when bound to growth factors. Activated RTKs stimulate the exchange of GTP for GDP on Ras by the Sos guanine nucleotide exchange factor (GEF), which is recruited from cytosol to cell membrane by an adaptor protein Grb2. RasGTP can directly interact with the Raf family kinases, and this interaction is the crucial first step of Raf activation. Activated Raf then triggers the phosphorylation cascade of MEK and ERK (reviewed in [8]). One of ERK's substrates is the p90 ribosome protein kinase (RSK). Activated ERK and RSK have been shown to phosphorylate TSC2 and activate mTORC1 by blocking the formation of TSC1/2 complex and inhibiting the ability to turn off Rheb, respectively [9, 10]. ERK can also promote mTORC1 activation by phosphorylating one of its key component, Raptor [11].

Ribosome protein S6 as an mTORC1 downstream molecule and biomarker

As a key controller of cell growth, survival and metabolism, mTORC1 has a broad panel of downstream effectors, including the 70KD S6 kinase (p70S6K). It was widely accepted that p70S6K mediated S6 phosphorylation controlled the translation of mRNAs containing an oligopyrimidine tract within their 5′ UTR (5'TOP mRNAs) [12], however, conflicting studies showed that translation of 5'TOP mRNAs is independent of p70S6K or phosphorylated S6 [13]. Although the role of S6 phosphorylation in tumorigenesis is unclear, it can still serve as a good readout for mTORC1 activity due to its high sensitivity to extracellular and intracellular environment changes. In the current study, we dissected the growth factor-driven PI3K and MAPK pathways in details to elucidate the usability of phospho-S6 as a biomarker in clinical trials.

Material and methods

Plasmids and virus production

pBabe-puro-p110αH1047R and pBabe-puro-BrafV600E plasmids were kind gifts from Dr Neal Rosen's lab. The p110αH1047R insert was then subcloned to the pBabe-neo vector from Addgene (Addgene plasmid 1767) using BamHI/SalI restriction sites. 293FT cells were cotransfected with ecotropic env packaging plasmid and either pBabe-neo-p110αH1047R or pBabe-puro-BrafV600E plasmid using Lipofectamine 2000 (Invitrogen). Virus-containing media was collected 48 hours after transfection and used to infect 3T3 cells in the presence of

polybrene (Millipore). Infected cells were selected with puromycin (3μg/ml) and G418 (500 μg/ml) to generate stable lines.

Cell culture and inhibitors

Cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum (Omega Scientific), 2 mM glutamine and 50 units/ml each of penicillin and streptomycin as suggested by the American Tissue Culture Center. Akti was obtained from Merck. PD0325901 was purchased from CalBiochem. RAD001 was obtained from Novartis. All compounds were dissolved in dimenthyl sulfoxide (DMSO).

Western blot and antibodies

Cells were washed with PBS buffer and lysed in M-PER mammalian protein extraction reagent (Thermo Scientific). Protein concentration was quantified using the Bicinchoninic Acid (BCA) assay. Same amount of total protein was resolved by SDS-PAGE and transferred to Immobilon membrane. Membrane was blocked in TBS-T with 5% fat-free milk and then incubated with specific primary and secondary antibodies. Signal was detected using ECL Plus reagent (Ambersham). Antibodies for p-Akt (S473), pAkt (T308), pERK, p-p70S6K(T389), pS6(S235/236), pS6(S240/244), p-pras40, and ERK were from Cell Signaling Technology.

Cell proliferation assay

3T3 sublines were seeded in 6-well plates at the density of 50,000/well in triplicates and treated with indicated drugs 24 hours after seeding. Cell number was counted on day 1 and day 4 using Vi-CELL (Beckman). MCF7 and HCT15 cells were seeded in 96-well plates at the density of

5000/well in triplicates and treated with indicated drugs 24 hours after seeding. Viable cells were measured using CellTiter-Glo luminescent assay (Promega).

Result

S6 phosphorylation regulation in genetically engineered cells

Overexpression of p110α^{H1047R} and Braf^{V600E} in 3T3 cells

To study the role of PI3K and MAPK pathways in phospho-S6 regulation alone, or in combination without the interference of genetic background difference, we overexpressed constitutively active mutants p110α^{H1047R} and Braf^{V600E} in 3T3 cells. Retrovirus was produced in 293FT cells by costransfection of ecotropic env packaging plasmid with pBabe-neo-p110αH1047R or pBabe-puro-BrafV600E plasmid. 3T3 cells were transduced with either virus alone or both. Empty vectors were used as negative control. Stable cell lines were generated by puromycin and neomycin selection. Overexpression of p110α^{H1047R} resulted in elevated phospho-Akt level while BrafV600E causes elevated phospho-ERK level, indicating the activation of PI3K/Akt and MAPK/ERK pathway, respectively. Both pathway activation lead to phospho-S6 upregulation and cell growth advantage, and the dual mutation combination had an additive effect (Figure 2-1).

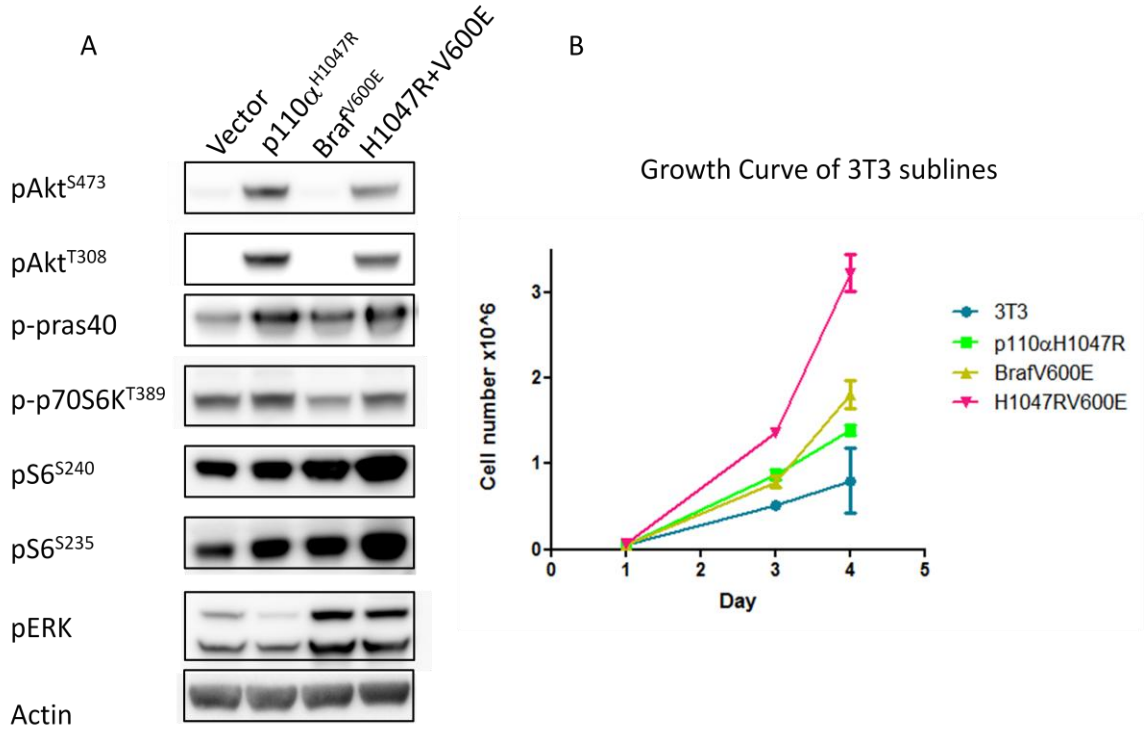

Fig 2-1 Overexpression of constitutively active mutant of p110α and Braf in 3T3 cells causes activation of PI3K and MAPK pathway respectively. A. Western blot of 3T3 stable lines. Cells were incubated in DMEM with 2% serum for 1h before lysed. B. Growth assay of 3T3 stable lines. 50,000 cells were seeded in triplicate in 6-well plate, incubated in DMEM with 2% serum and counted on day1, 3 and 4. Data are shown as Mean ± S.D.

Drug treatment of transformed 3T3 cells

To ask if the upregulation of phospho-S6 by either mutant was pathway specific, we used the highly selective inhibitors of Akt (referred to as Akti) and MEK (PD0325901, referred to as PD901) to block each pathway. Akti is a PH-domain-dependent inhibitor of Akt1 and Akt2, with no inhibition of other AGC kinases [14]. PD901 is a non-ATP-competitive inhibitor of MEK1

and MEK2 with an IC_{50} of 1nM [15]. Akti significantly downregulated phospho-S6 level in $p110\alpha^{H1047R}$ transformed cells while PD901 decreased phospho-S6 level in $Braf^{V600E}$ transformed cells. However, in cells transformed with both mutants, phospho-S6 level was inhibited only with the combination treatment of Akti and PD901 (Fig 2-2), suggesting that S6 being a common downstream target of both PI3K and MAPK pathways.

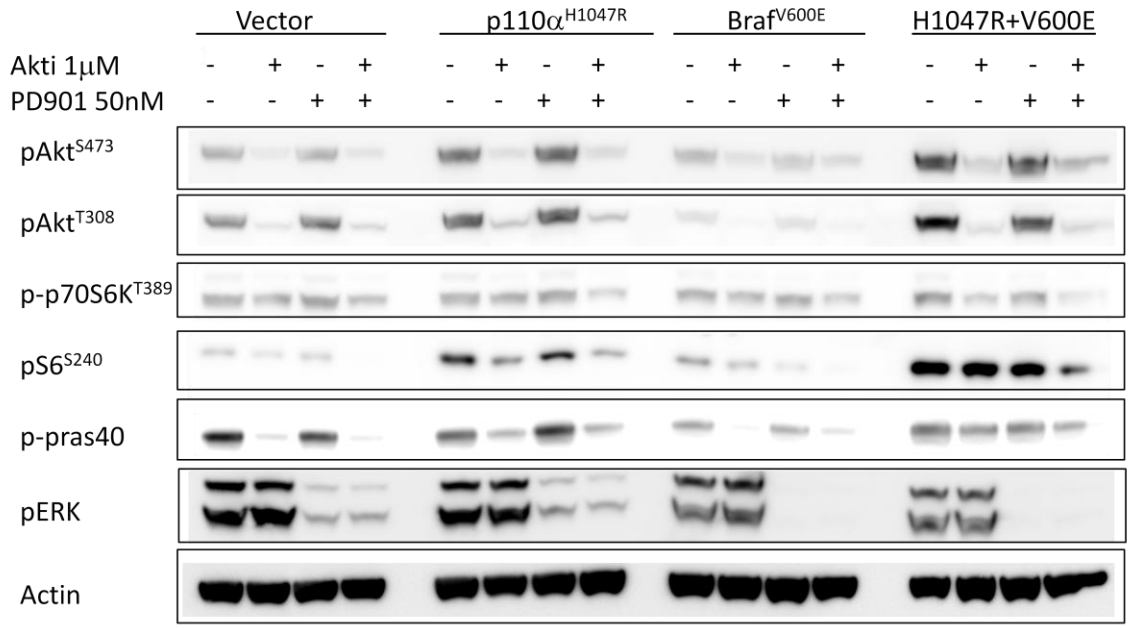

Fig 2-2 S6 phosphorylation was regulated by both PI3K and MAPK pathways. 3T3 stable cell lines were treated for 6h with indicated drugs in fresh DMEM with 2% serum, and then lysed for western blot.

We also examined the effect of the inhibitors on cell proliferation (Fig 2-3). 3T3 p110αH1047R cells were sensitive to Akti but resistant to PD901, while BrafV600E cells were sensitive to PD901. The minor inhibitory effect of Akti on BrafV600E cells suggested that Braf, like Ras, might activate the PI3K/Akt pathway [16]. H1047R/V600E double mutated cells responded to both Akti and PD901, but only with the combination treatment was the proliferation rate brought back to that of the control cells. Neither Akti nor PD901 had any significant inhibitory effect in 3T3 control cells, which suggested that the drug response was not due to general toxicity.

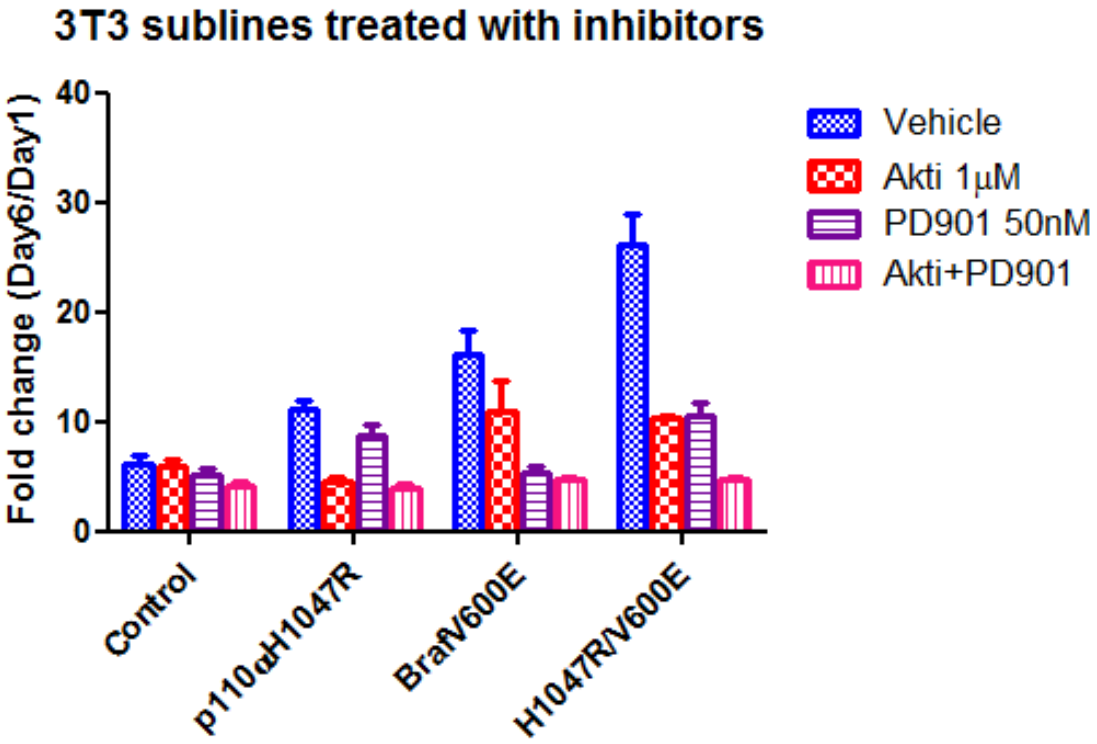

Fig 2-3 Growth advantage gained by constitutively active pathways was reversed by pathway specific inhibitors. 50,000 cells of each stable line were plated in triplicate and treated with indicated drugs. Cell numbers were counted on day 1 and day6. Data are shown as Mean ± S.D.

mTORC1 has been implicated as a main downstream factor of the PI3K/Akt pathway, whose activation leads to the phosphorylation of S6. To test whether mTORC1 regulated S6 phosphorylation when both PI3K and MAPK pathways were activated, we treated the 3T3 cell lines with a rapamycin analog (rapalog), RAD001. Not only was S6 phosphorylation sensitive to RAD001 when the PI3K or MAPK pathway was activated alone, but also when both pathways were upregulated, suggesting that both pathways stimulated S6 phosphorylation via mTORC1 (Fig 2-4).

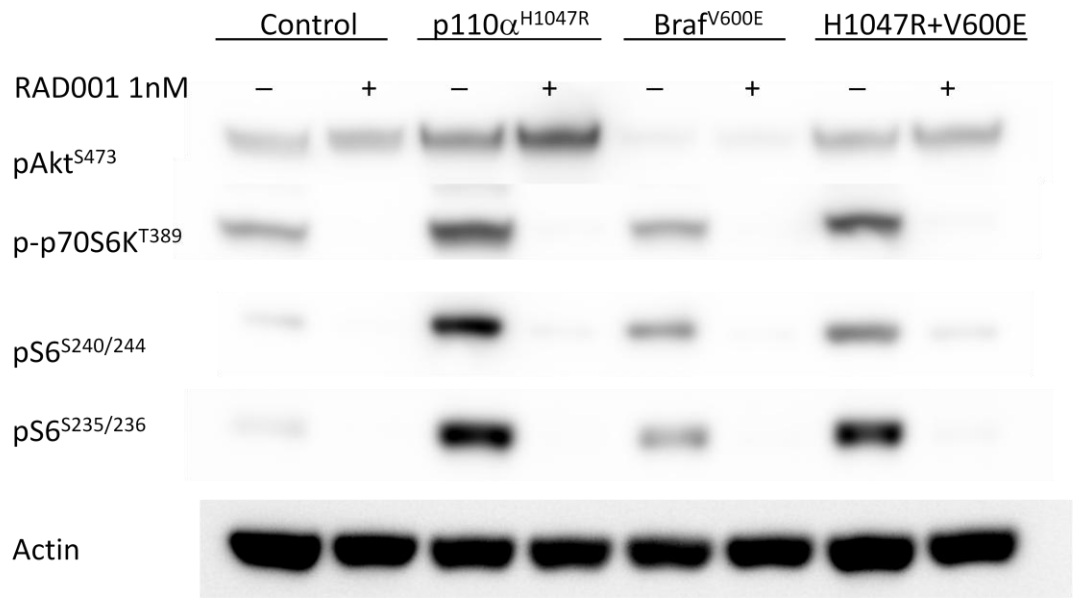

Fig 2-4 RAD001 inhibited S6 phosphorylation when both PI3K and MAPK pathways were activated. Cells were treated with RAD001 for 1h before lysed for western blot.

S6 phosphorylation regulation in cancer cells

Overactivation of PI3K and/or MAPK pathways is commonly seen in human cancers. To determine whether phosphorylation of S6 was regulated by either or both pathways in real diseases in the same pattern as the genetically engineered cells, we treated a panel of human

cancer cell lines with the pathway specific inhibitors. In cell lines with either p110α mutation or PTEN loss, S6 phosphorylation was sensitive to Akti alone while ERK phosphorylation level was not affected (Fig 2-5A). In cell lines with both p110α mutation and RAS mutation, S6 phosphorylation was resistant to Akti treatment alone (Fig 2-5B). However, RAD001 inhibited S6 phosphorylation in all cell lines. These results were all in agreement with the genetically 3T3 cell lines, which suggested that the transformed 3T3 system could be a useful tool to study pathway signaling in disease.

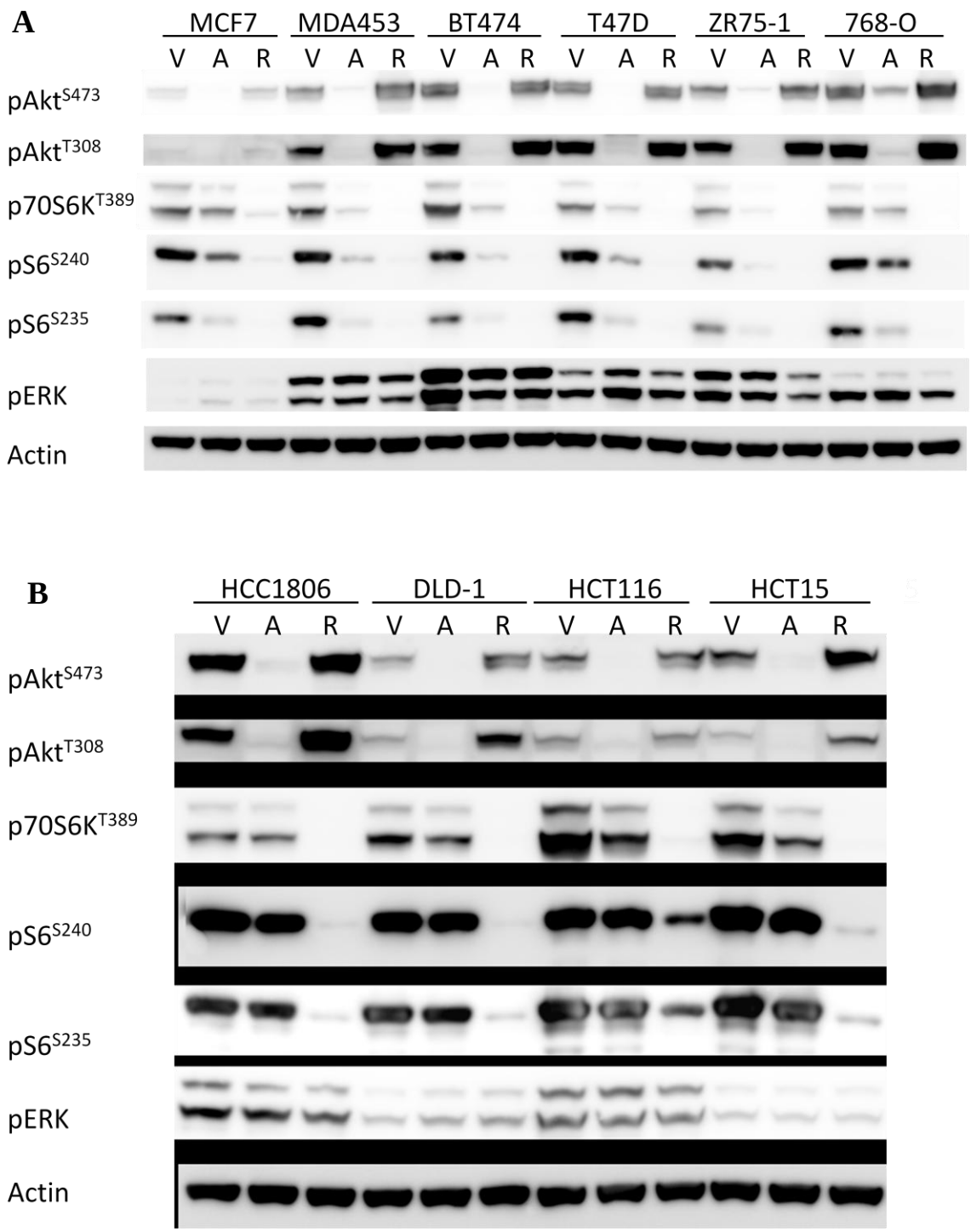

Fig 2-5 S6 phosphorylation in cancer cell lines was sensitive to Akti when only the PI3K pathways was overactivated (A), but resistant to Akti when both PI3K and MAPK pathways

were active (B). Cells were replenished with fresh media with indicated drugs for 6h before lysed for western blot. V – Vehicle; A – Akti 1μM; R – RAD001 1nM.

Next, we took MCF7 and HCT15 as the example of cancer cell lines with p110α mutation alone or concurrent p110α/KRAS mutations, respectively, to further study the regulation of S6 phosphorylation by each pathway. Phospho-S6 level was inhibited by 1μM Akti alone in MCF7 cell, and was not further downregulated by the combination of Akti and PD901. Consistently, MCF7 cell proliferation showed sensitivity to Akti alone. Contrastingly, in HCT15 cells neither phospho-S6 nor phospho-p70S6K was inhibited by Akti or PD901 alone, despite the effective inhibition of phospho-Akt and phospho-ERK level. HCT15 also was resistant in cell proliferation to either single reagent. Only with the combination of Akti and PD901 was the proliferation rate significantly decreased (Fig 2-6). Notably, RAD001 only had a relatively minor effect in cell proliferation despite nearly complete inhibition in phospho-S6 in both cell lines. This lack of correlation between inhibition of phosphoS6 and proliferation might be explained by the induction of autophagy caused by mTORC1 inhibition, as discussed further below [17].

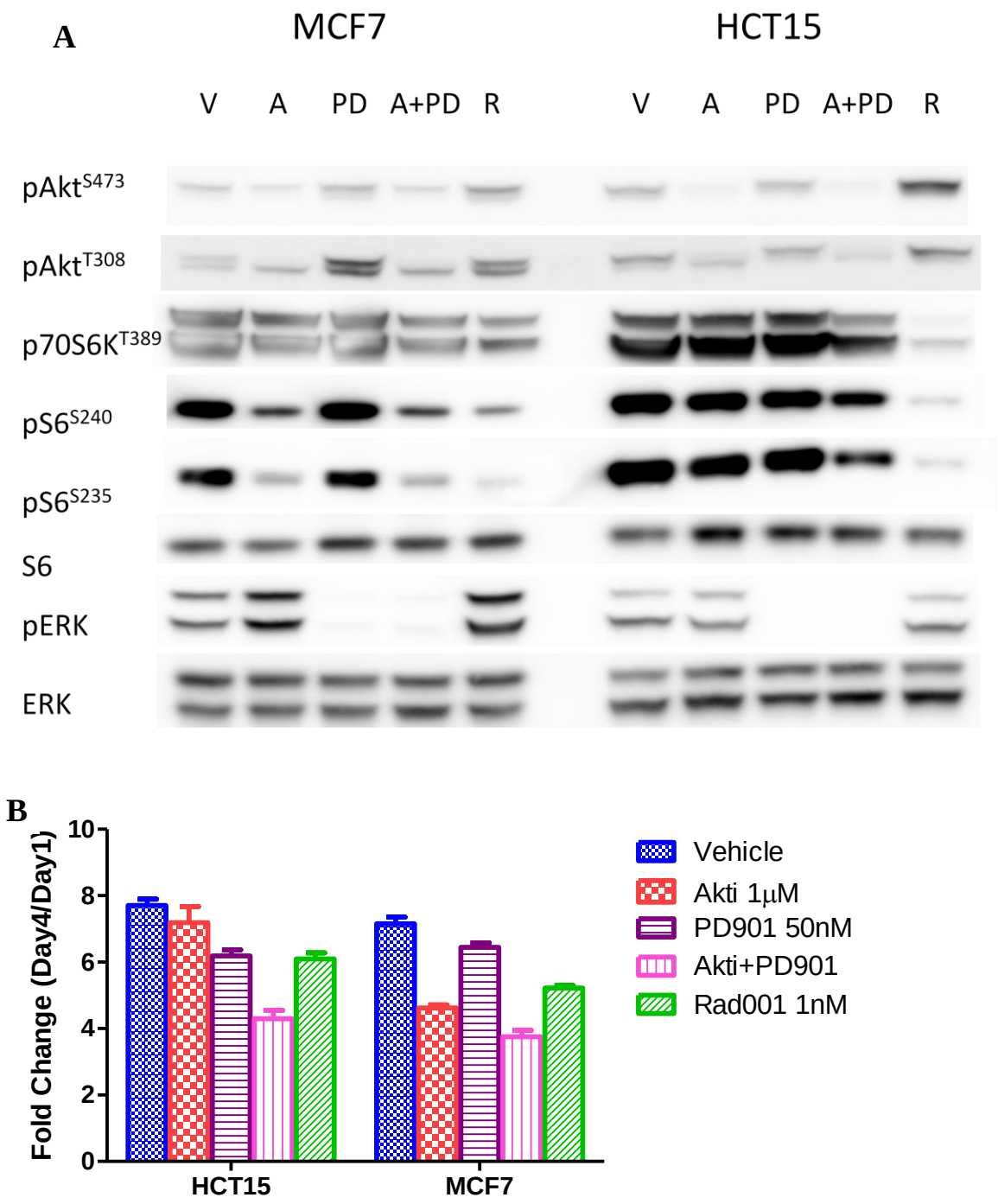

Fig 2-6 MCF7 cell was sensitive to Akti and HCT15 was resistant to either Akti or PD901. A. Western blot of cells treated with drugs. Cells were replenished with fresh media with indicated drug(s) for 6h before lysed for western blot. V – vehicle; A – Akti 1mM; P – PD901 50nM; R –

RAD001 1nM. B. Cell proliferation was assessed using the Cell Titer-Glo luminescent assay after 4 days of treatment with indicated drugs.

Discussion

With the rapid progression in small molecule cancer drug development, the use of proper biomarkers becomes more and more indispensable to patient selection and clinical impact evaluation [18]. S6 phosphorylation has been widely used as the readout of PI3K/Akt/mTOR pathway in various clinical trials [19-21]. However, more and more studies have shown that phospho-S6 regulation is the result of multi-pathway networking, among which are the PI3K/AKT and RAS/Raf/MEK/ERK pathways [22-24]. In the current study, we demonstrated that phospho-S6 was regulated by either or both pathways under different conditions, mainly depending on the mutation status of the cells.

S6 is predominantly phosphorylated by p70S6K, in somatic cells under the control of mTORC1, which conveys signals from PI3K/Akt [25, 26]. Our data showed that overactivation of the PI3K/Akt pathway by either PTEN loss or p110α mutation further sensitized S6 phosphorylation to Akt inhibition (Fig 2-2 and 2-5A), suggesting signaling dependence in cancer cells. However, the dependence was lost with the coexistence of a constitutively active Ras or Braf mutant (Fig 2-2 and 2-5B), indicating that when overactivated, MAPK pathway can compensate for PI3K pathway in S6 upregulation. More importantly, proliferation rate of such cells was inhibited only with the usage of both Akti and PD901, underlining the importance of combination treatment in

selected patients, since concurrent PI3K/MAPK overactivation is a common event in human cancers [27, 28].

mTOR has been identified as the central controller of cell growth [29], and aberrantly high activity of mTOR as well as its upstream and downstream signaling components appears to be the underlying cause of numerous cancers [28, 30-32]. Hence, rapalogs have been widely used in dissecting the mechanisms of tumorigenesis and development. However, rapalogs in anticancer clinical trials have variable results, showing promise against only a few cancers [33-35]. Our data showed that one rapalog, RAD001, had modest effect in cell growth inhibition despite the excessive downregulation of mTORC1 downstream effectors, including phospho-S6.

One model to explain this discrepancy is the pro-survival role of autophagy in cancers [36-38]. Autophagy is a catabolic process of intracellular protein and organelle degradation via lysosomes. It is highly induced when cells are under metabolic stress, such as starvation and hypoxia [39, 40]. When prolonged, autophagy will eventually lead to cell death [41]. However, it can also serve as a temporary survival mechanism by providing cells with an alternative source of energy. Cancer cells often experience stress due to poor angiogenesis and overcrowded population, and autophagy promotes cancer cell survival under such conditions and mediates resistance to anticancer therapy [42, 43]. The key components of mammalian autophagy machinery is a large complex composed of the unc-51-like kinases 1 and 2 (ULK1/2), the mammalian homolog of autophagy-related gene 13 (mAtg13), and the scaffold protein FIP200 [44, 45]. mTORC1 is a major checkpoint of autophagy induction or suppression [29] by integrating signaling from

PI3K/Akt pathway and sensing nutrient and energy status of cells. When the environment is rich, active mTORC1 is associated with and phosphorylates the ULK1/2-mAtg13-FIP200 complex. Under nutrient-deprived conditions, mTORC1 dissociates from the complex, which is then partially dephosphorylated and activates the autophagy cascade [46]. Rapalogs induce autophagy by inhibiting mTORC1 activity, which might explain how certain cancers are resistant to the drug. However, rapalogs have also been implicated to sensitize cancer cells to radiation or oncolytic adenovirus therapy via induced autophagy [47, 48]. The detailed mechanism of autophagy's prosurvival and prodeath dual function is still ambiguous. One plausible model is the balance between maintenance of cellular homeostasis and overconsumption of cellular components. Autophagy is cytoprotective by removing dysfunctional or damaged organelles which are digested and recycled for cellular metabolic needs. Conversely, when cellular degradation reaches a crucial point that exceeds new synthesis, autophagic cell death occurs. More studies need to be conducted to determine whether autophagy inducers or inhibitors may be useful in specific anticancer treatment. One strategy to reach this goal is to preselect patients checking different biomarkers such as PTEN status and oncogenic kinase mutations [48, 49]. Phospho-S6 may serve as a readout for basal level mTORC1 activity in patient samples since it is sensitive to both PI3K and MAPK pathway signaling, as well as nutrient input.

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Chapter 3

Regulation of S6 ribosome protein phosphorylation by amino acids

Introduction

mTORC1 receives multiple upstream signals including growth factors, nutrients (especially amino acids), energy and stress [1, 2]. It is understandable that S6, as a main downstream effector of mTORC1, is also regulated by the above signals. In this part of the study, we focused on amino acid-mediated mTORC1/S6 regulation.

Amino acids facilitate the recognition of mTORC1 and its substrates [3, 4]. However, unlike the well documented PI3K/Akt/mTOR and Ras/ERK/RSK/mTOR pathways, the detailed mechanism of amino acid-mediated mTORC1 activation is still largely unclear. Although the Class III PI3K, hVps34, has been implicated in the nutrient respsonse of mTORC1, its precise function still needs to be established [5, 6]. A *Ste20*-family kinase, MAP4K3, has also been shown to regulate mTORC1 activity promoted by amino acids [7], but the mechanism of its action on mTORC1 is yet to be defined. Lately, both functional screens and biochemical purification have identified the Rag GTPases as a key component in amino acid-mediated mTORC1 activation [8, 9]. Rag GTPases serve as a docking site for mTORC1 on the surface of lysosome [10]. There are four Rag GTPases (RagA, RagB, RagC and RagD) which function as heterodimers. RagA and RagB are functionally redundant and active when bound to GTP. RagC and RagD are also functionally redundant and active when bound to GDP. RagA or RagB forms a heterodimer with either RagC or RagD., The RagB-GTP/RagC-GDP dimer recovered the highest amount of mTORC1 in co-immunoprecipatation assay and showed stronger effect than their wildtype counterpart in activating mTORC1 in the absence of amino acids. In contrast, RagB-GDP/RagC-GTP was not found associated with mTORC1 [9]. The heterodimer is

constantly localized to lysosome by interacting with a trimetric protein complex called Ragulator [10]. When cells are deprived of amino acids, the Rag heterodimer is inactive and does not bind mTORC1, which is diffuse throughout the cytosplasm. In the presence of amino acids, the Rag heterodimer is activated by flipping the bound nucleotides. Active Rag heterodimers then recruit mTORC1 to the surface of lysosome where it is activated by the lysosomal pool of Rheb (see Fig 3-8 in discussion). A constitutively GTP-bound RagB mutant can eliminate the necessity of amino acids in mTORC1 activation while a constant GDP-bound RagB abolishes mTORC1 activity even in the presence of amino acids or insulin [9]. Therefore, Rag GTPases are necessary for amino acid-mediated mTORC1 activation. In the current study, we used the RagB-GTP mutant to explore the amino acid-induced S6 phosphorylation and its relationship with PI3K/MAPK pathway signaling.

Material and methods

Plasmids and virus production

Flag pLJM1 RagB 99L (referred to as RagB GTP) was purchased from Addgene (Plasmid 19315). 293FT cells were cotransfected with amphotropic env packaging plasmid and RagB GTP plasmid using Lipofectamin 2000 (Invitrogen). Virus-containing media was collected 48 hours after transfection and used to infect MCF7 cells in the presence of polybrene (Millipore). Infected cells were selected with puromycin (1μg/ml). In transient expression experiment, 293FT cells were transfected with RagB wildtype or RagB-GTP plasmid only.

Cell culture and conditioned media

Cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum (Omega Scientific), 2 mM glutamine and 50 units/ml each of penicillin and streptomycin as suggested by the American Tissue Culture Center. Serum-free amino acid-free media were made by in-house facility. Insulin, 50x amino acid mix and metformin were purchased from Sigma Aldrich. Epidermal growth factor (EGF) was a generous gift from Dr. Ingo Mellinghoff's lab

Western blot and cell proliferation assay

Described in Chapter 2.

Results

S6 phosphorylation regulation in growth-factor or nutrient-deprived conditions

Growth factors and nutrients are both key components in the cancer microenvironment, as they not only activate intracellular signaling pathways that are important for tumor progression, but the presence or absence of certain molecules may even affect anticancer treatment [11]. There has been growing interest focusing on how the microenvironment influences cancer cells. Since it is quite common that growth factors and nutrients are depleted in the tumor microenvironment, we used media lacking either growth factors or nutrients, or both, to better understand the regulation of phospho-S6 in such conditions.

We treated two colorectal cell lines (HCT15 and HCT116) with kinase inhibitors in media that has been in tissue culture for 48 hours to mimic the growth factor and nutrient depleted tumor microenvironment (referred to as depleted media). We treated cell lines with same drugs but in fresh media as control. Both HCT15 and HCT116 harbor p110α/KRas dual mutations and, as expected from the data in chapter 2 (Fig 2-5B), phospho-S6 was not reduced by Akti alone in fresh media. However, phospho-S6 in both cell lines was reduced by Akti in depleted media where growth factor and nutrient level was low (Fig 3-1). This data suggested that lack of either growth factors or nutrients, or both, sensitized phospho-S6 in cancer cells to Akt dependence.

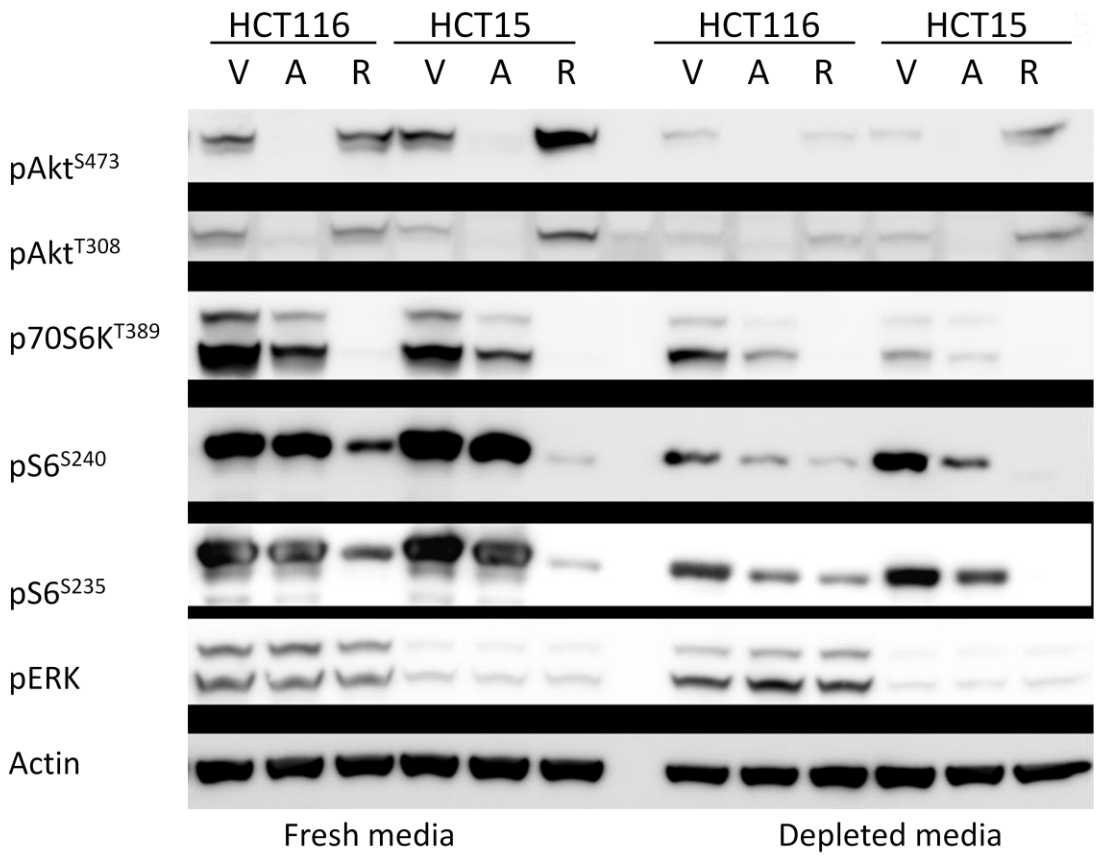

Fig 3-1 S6 phosphorylation in HCT15 and HCT116 cells was sensitized to Akti when both growth factors and nutrients were poor. Cells were maintained in regular growth media for 48h before treatment. One set of cells were replenished of fresh media with indicated drug. Drug was added directly into media of the other set of cells. Cells were treated for 6h before lysed for western blot. V – vehicle, A – Akti 1μM, R – Rad001 1nM

To answer the question whether it was growth factors or nutrients that caused the shift in sensitivity of S6 phosphorylation to Akt inhibition, we treated HCT15 cells with kinase inhibitors in starvation media (no serum, no amino acids) and then stimulated with different growth factors or nutrients one at a time. Phospho-S6 was inhibited by either Akti or PD901 in starvation media. When cells were stimulated with insulin, phospho-S6 as well as phospho-Akt level was significantly elevated, and inhibited by Akti treatment but not PD901, suggesting that insulin stimulated S6 phosphorylation via PI3K/Akt pathway. Epidermal growth factor (EGF) increased phospho-S6 and phospho-ERK level in the cells, and PD901 but not Akti treatment reversed the effect by inhibiting the MAPK pathway. These data suggested that lack of growth factors was not the cause for sensitivity change of phospho-S6 regulation by pathway specific inhibitors in starvation media. In contrast, amino acids potently induced p70S6K and S6 phosphorylation in starved cells but had little effect on phospho-Akt or phospho-ERK level, and neither Akti nor PD901 inhibited the amino-acid-mediated S6 phosphorylation (Fig 3-2). The fact that RAD001 abolished phospho-S6 in all conditions suggested that both growth factors and nutrients upregulated S6 phosphorylation through mTORC1, but nutrients acted in a PI3K/MAPK- independent manner.

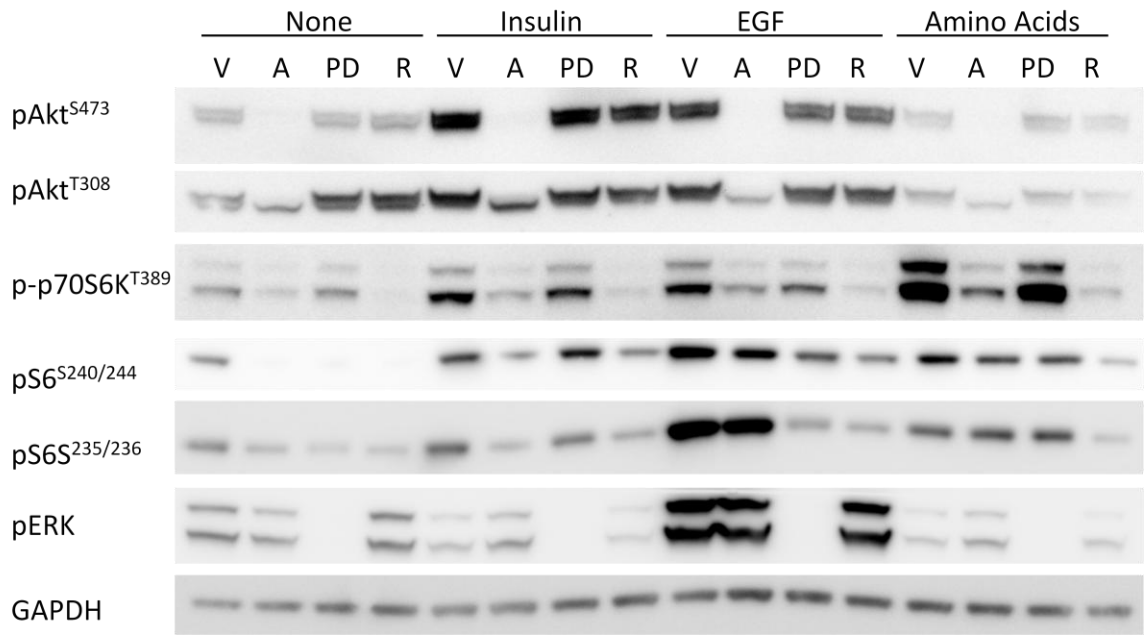

Fig 3-2 Nutrients upregulated S6 phosphorylation in a PI3K/MAPK-independent manner. Cells were washed and replenished with fresh media with no serum or amino acids with indicated drugs. Cells were treated for 50 minutes and then stimulated with different compounds for 5 minutes before lysed for western blot. V – vehicle; A – Akti 1mM; P – PD901 50nM; R – RAD001 1nM.

S6 phosphorylation regulation by amino acids mediated by Rag/Rheb/mTORC1 GTPases

Since Rag GTPases have been shown to be a key component in amino acid-mediated mTORC1 signaling, we used a constitutively active form of one of the GTPases, RagB-GTP, to explore the role of amino acids in S6 phosphorylation and its potential as a therapeutic target in anticancer treatment.

When overexpressed in 293FT cells via transfection, RagB-GTP not only increased the basal level of phospho-S6, but also made the cells insensitive to amino acid withdrawal and the following amino acid stimulation (Fig 3-3).

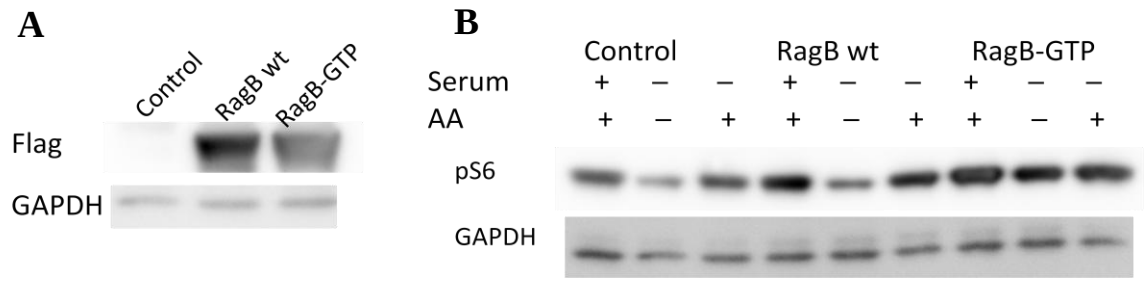

Fig 3-3 Overexpression of RagB-GTP in 293FT cells caused loss of regulation of phospho-S6 by amino acids. A. Western blot of 293FT cells transfected with vector control, Flag-RagB wildtype (wt) or Flag-RagB-GTP. B. Western blot of transfected 293FT cells. Cells were washed with and incubated in serum-free amino-acid-free media for 50 minutes then stimulated with either serum (10% volume) or 50x amino acids (AA) mix for 10 minutes before lysed for western blot.

To further elucidate the relationship between amino acid- and growth factor-input to mTORC1/S6 regulation, we generated a stable MCF7 cell line that overexpressed RagB-GTP and treated these cells with PI3K/MAPK pathway specific inhibitors. Phospho-S6 levels were insensitive to Akti in MCF7 cells expressing RagB-GTP but still responsive to RAD001 (Fig 3-4), showing that amino acid-induced mTORC1 activation was sufficient to upregulate S6 phosphorylation independent of Akt.

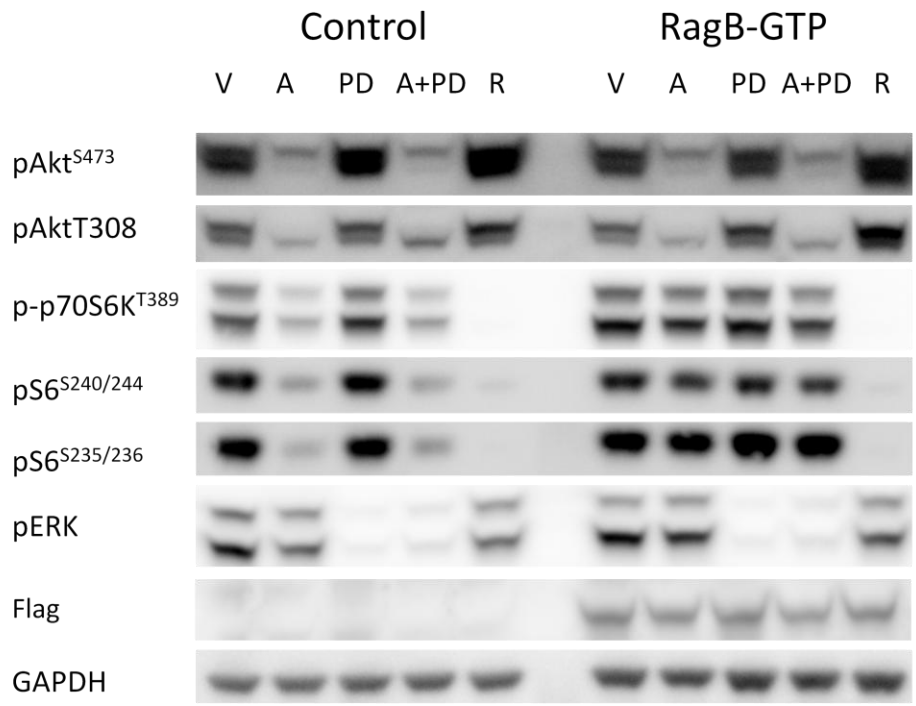

Fig 3-4 Constitutively active Rag-GTP-mediated mTORC1 caused resistance to PI3K pathway inhibition. A. Western blot of MCF7 cells treated with drugs. Cells were replenished of fresh media with indicated drug and treated for 6h before lysed for western blot.

We then asked the question whether this constitutive activation of the amino-acid input pathway to S6 (via Rag proteins) had any biological effect. RagB-GTP expressing cells had a growth advantage compared to control cells in cell proliferation, and the effect was more evident in the late stage of the assay as most nutrients had been consumed by proliferating cells(Fig 3-5). This gain of function is presumably because RagB-GTP expressing cells were more resistant to nutrient-deprived condition than control cells.

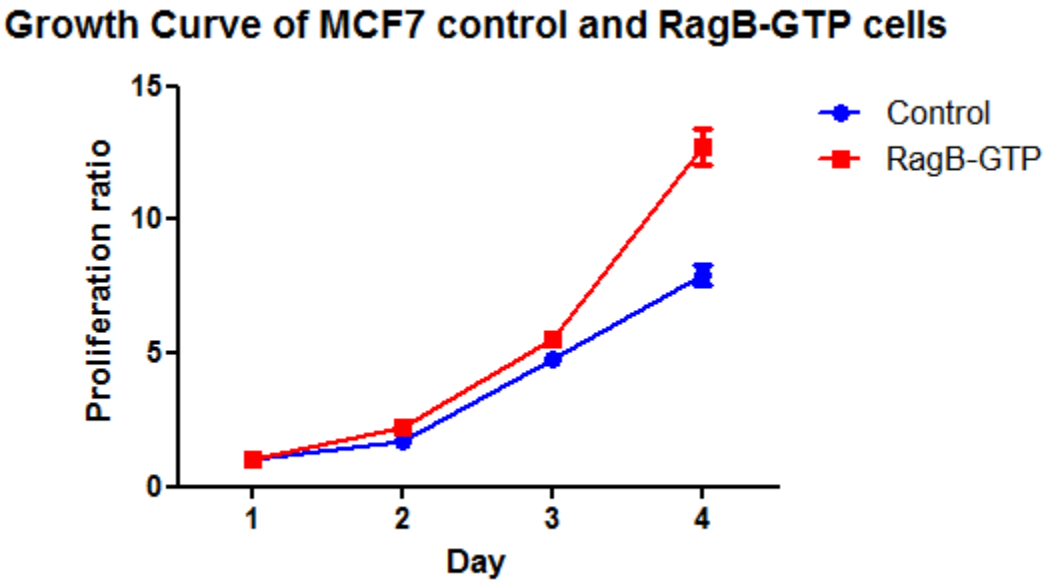

Fig 3-5 Cell proliferation assay of MCF7 control cells and Rag-GTP stable cells. Cells were plated in 96-well plate in triplicates at the density of 5,000cells/well and cell proliferation was assessed by CellTiter-Glo luminescent assay on Day 1, 2 and 4. Data are shown as Mean ± S.D.

Since our data suggested that constitutive upregulation of S6 phosphorylation by amino acids be converted to growth advantage in cancer cells, the next question was whether inhibiting amino-acid-mediated S6 phosphorylation could potentially facilitate anticancer treatment. It is impossible to completely eliminate amino acids in cells partly due to protein degradation and amino acid recycling. Hence, we used metformin, a drug reported to block Rag GTPase-mediated mTORC1 signaling, to mimic the amino acid-free condition and explore the amino acid-induced S6 phosphorylation as a targetable liability in anticancer treatment. Metformin is a widely used drug to treat type 2 diabetes patients by activating AMP-activated kinase (AMPK). Recently it was reported to phenocopy amino acid withdrawal through Rag GTPases independent of AMPK as further described below in discussion [12]. In our study, metformin significantly inhibited S6 phosphorylation in MCF7 control cells but not in RagB-GTP cells (Fig 3-6A), indicating that RagB-GTP rescues the inhibitory effect of metformin. The induction of phospho-Akt by metformin in control cells can be explained by removal of the negative feedback loop mediated by mTORC1/p70S6K [13]. Metformin also inhibited MCF7 cell proliferation, whereas RagB-GTP expressing cells were relatively resistant (Fig 3-6B), suggesting that blocking amino acid-induced mTORC1/S6 signaling could potentially facilitate anticancer treatment.

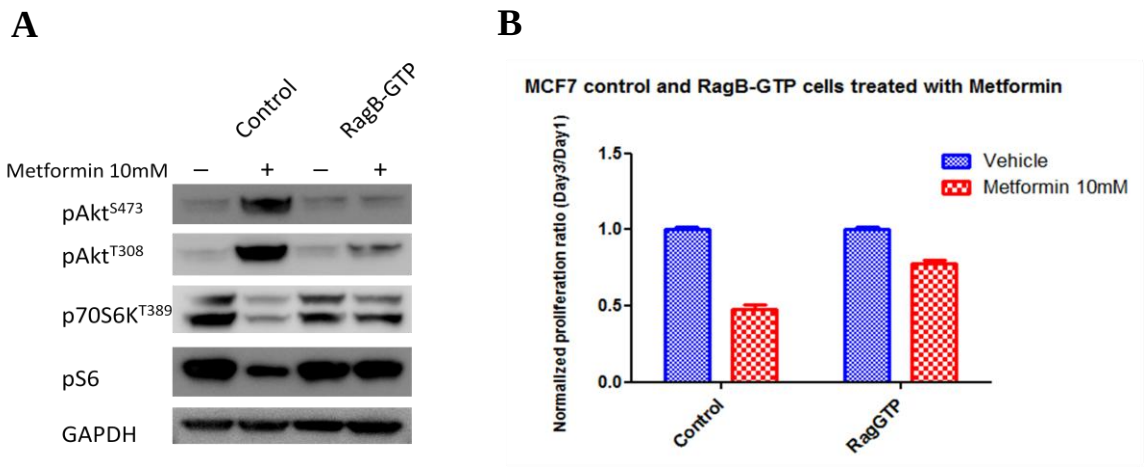

Fig 3-6 Metformin inhibited S6 phosphorylation and cell proliferation by blocking Rag GTPase-mediated mTORC1 signaling. A. Western blot of MCF7 RagB-GTP cells. Cells were treated with metformin (10mM) for 24h before lysed for western blot. B. Cell proliferation assay of MCF7 RagB-GTP cells treated with metformin. Cells were seeded in 96-well plate in triplicates at the density of 5000cells/well and treated with 10mM metformin (final concentration). Cell proliferation was assessed by CellTiter-Glo luminescent assay on Day 1 and 3 and normalized to vehicle treated cells. Data are shown as Mean ± S.D.

To test the hypothesis that amino acid-mediated mTORC1 activation could be a therapeutic target, we treated the p110α/KRas dual mutation cancer cell line HCT15 with the combination of metformin and PI3K/MAPK pathway specific inhibitors. Metformin not only inhibited HCT15 cell proliferation by itself, but also further enhanced the inhibitory effect of Akti and PD901 combination treatment (Fig 3-7), suggesting that blocking amino acid-induced mTORC1 activation could be a therapeutic strategy applied to a broad spectrum of cancers regardless of their PI3K/MAPK mutation status.

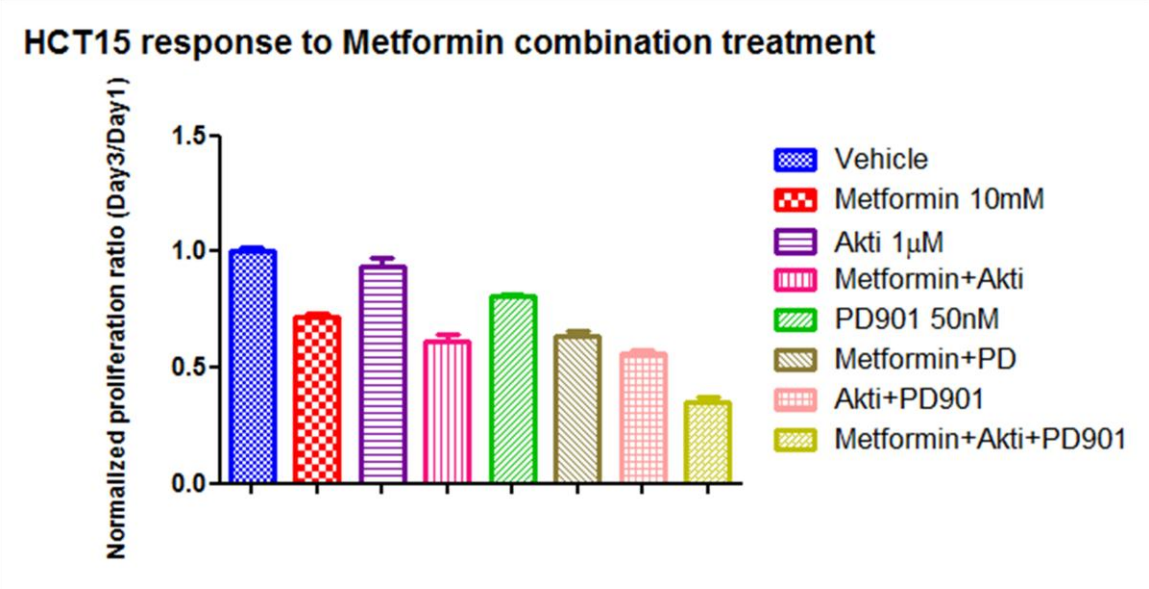

Fig 3-7 Metformin facilitated growth inhibition by PI3K/MAPK pathway inhibitors in HCT15 cells. Cells were seeded in 96-well plate in triplicates at the density of 5000cells/well and treated with indicated drugs. Cell proliferation was assessed by CellTiter-Glo luminescent assay on Day 1 and 3 and normalized to vehicle treated cells. Data are shown as Mean ± S.D.

Discussion

mTORC1 integrates multiple upstream regulators and conveys signals to its downstream effectors, including the ribosome protein S6 [14]. In the previous chapter, we demonstrated that phospho-S6 was under the control of growth factor driven-PI3K/MAPK pathways in different genetic backgrounds. In this chapter, we showed that amino acids regulated S6 phosphorylation in a broader spectrum and moreover, provided a therapeutic strategy to target the amino acid/mTORC1/S6 signaling cascade in anticancer treatment.

Rheb is the common signaling mediator to mTORC1 from growth factors and amino acids, and overactivation of Rheb can induce oncogenic transformation [15, 16]. But growth factors and amino acids act through distinct mechanisms. Growth factors such as insulin activate Rheb (via the PI3K/AKT pathway) by removing the GAP activity of the TSC1/2 complex [17, 18], and sequentially increasing the GTP charging of Rheb [19, 20]. On the other hand, amino acids bring mTORC1 to the proximity of Rheb on the surface of lysosome via the Rag GTPases and facilitates the interaction of mTORC1 and Rheb[10]. Rheb binding of mTORC1 does not require Rheb guanyl nucleotide charging, but it activates mTORC1 in a GTP-dependent manner [21]. In the current study, we used a constitutively active Rag GTPase to show that amino acid-mediated mTORC1 activation was sufficient to induce S6 phosphorylation even in the presence of PI3K/MAPK pathway inhibitors, and further showed that growth factors and amino acids upregulate mTORC1 activity and its substrates via distinct mechanisms.

Cancer cells can survive and even outcompete their neighboring normal cells in adverse conditions due to hyperactivated growth factor-driven pathways and highly efficient amino acid recycling. Recent discoveries of Rag GTPase-mediated mTORC1 activation by amino acids not only provided insights into the field of metabolism, but also provided novel potential drug targets in anticancer treatment [22]. Our metformin data showed that pharmacologically blocking the Rag-mTORC1 interaction potently inhibited cancer cell proliferation.

Metformin belongs to the biguanides family of antidiabetic drug, whose main effect includes decreasing hepatic glucose output and increasing glucose uptake in peripheral tissue [23, 24]. The widely accepted model is that metformin exerts its action against diabetes by inhibiting mitochondrial respiratory chain complex I, which reduces ATP production and activates AMPK. Activation of AMPK has been considered to be the mechanism of mTORC1 inhibition in metformin treated patients. A retrospective record-linkage case-control study showed that diabetic patients treated with metformin had reduced risks of certain cancers [25], which is consistent with the model that AMPK negatively regulates Rheb in energy starvation conditions by phosphorylating TSC2 [26], and caused mTORC1 inhibition [27]. However, a recent study showed that metformin inhibited mTORC1 activity in AMPK knockout mouse embryonic fibroblasts (MEFs) as well as wildtype MEFs. Interestingly, metformin treatment phenocopied amino acid withdrawal in mTORC1 localization without interfering the steady state of amino acid [12]. Our data supported this model by showing that a constitutively active RagB mutant caused resistance to metformin at both biochemical and biological levels (Fig 3-6). Metformin further inhibited cell proliferation when combined with Akti and PD901, suggesting that effect of

blocking growth factor-driven pathway and amino acid-mediated signaling to mTORC1 was additive. More importantly, the fact that metformin inhibited both MCF7 (activated PI3K pathway) and HCT15 (activated PI3K/MAPK pathways) cells suggested that it could be used in a broader range of cancers, regardless of their mutation status.

Conclusions

To summarize, our data elucidated the detailed regulation mechanism of S6 phosphorylation by growth factor-driven pathways (Chapter 2) and amino acid-mediated signaling (Chapter 3). Growth factors activate mTORC1/S6 by increasing GTP charging of Rheb and amino acid-activated Rag GTPases bring mTORC1 to the proximity of Rheb (Fig 3-8).

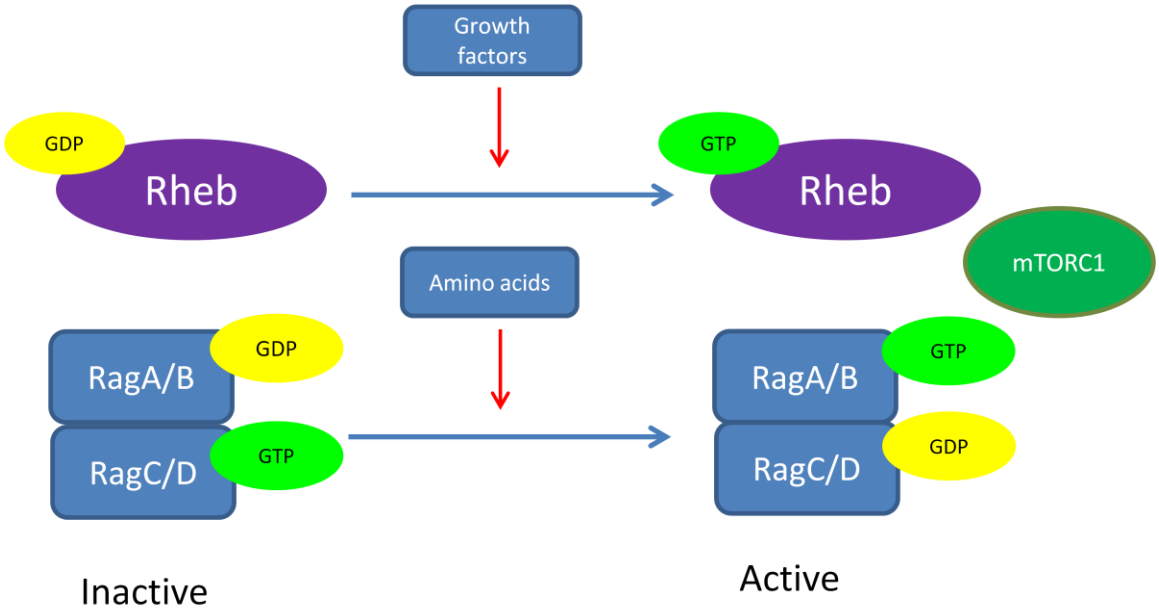

Fig 3-8 Growth factors and amino acids regulate mTORC1 via distinct pathways.

Our data also challenge the common notion that phospho-S6 is a good readout for the activity of the PI3K/Akt pathway. Instead, phospho-S6 reflects input from multisignal networking. The lack of correlation between phospho-S6 inhibition and cell proliferation in RAD001 treated cells further suggests that one biomarker is not sufficient in determining drug response. However, this does not exclude the use of phospho-S6 as a biomarker in anticancer treatment. Together with

other biomarkers such as Ki-67 staining and TUNEL assay, phospho-S6 can potentially facilitate patient selection and drug evaluation. For instance, pre-treatment phospho-S6 level together with cancer markers such as PTEN can be used to select patients to enroll in clinical trials [28] and determine their therapeutic strategies. Patients with one mutated pathway (PI3K or MAPK) can be treated with one specific kinase inhibitor (Akti or PD901 respectively). Patients with dual pathway mutations should be treated with both drugs. Phospho-S6 can be used to monitor their drug response because it may start showing changes before any clinical manifestation due to its high sensitivity to kinase inhibitors. For those patients whose phospho-S6 level is not decreased as being predicted by their mutation status, combination treatment with drug like metformin to target the amino acid axis should be in consideration.

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Chapter 4

Constitutively active androgen splice variant expressed in castration-resistant prostate cancer require full-length androgen receptor

Philip A. Watson, Yinan F. Chen, Minna D. Balbas, John Wongvipat, Nicholas D. Socci, Agnes Viale, Kwanghee Kim, Charles L. Sawyers

Abstract

Androgen receptor (AR) splice variants lacking the ligand binding domain (ARVs), originally isolated from prostate cancer cell lines derived from a single patient, are detected in normal and malignant human prostate tissue with the highest levels observed in late stage, castration-resistant prostate cancer. The most studied variant (called AR-V7 or AR3) activates AR reporter genes in the absence of ligand and could therefore play a role in castration resistance. To explore the range of potential ARVs, we screened additional human and murine prostate cancer models using conventional and next generation sequencing technologies and discovered several new, structurally diverse AR isoforms. Some, like AR-V7/AR3, display gain-of-function whereas others have dominant interfering activity. We also find that ARV expression increases acutely in response to androgen withdrawal, is suppressed by testosterone and, in some models, is coupled to full-length AR (AR-FL) mRNA production. As expected, constitutively active, ligand-independent ARVs such as AR-V7/AR3 are sufficient to confer anchorage-independent (*in vitro*) and castration-resistant (*in vivo*) growth. Surprisingly, this growth is blocked by ligand binding domain targeted antiandrogens such as MDV3100 or by selective siRNA silencing of AR-FL, indicating that the growth promoting effects of ARVs are mediated through AR-FL. These data indicate that the increase in ARV expression in castrate-resistant prostate cancer is an acute response to castration rather than clonal expansion of castration or antiandrogen-resistant cells expressing gain-of-function ARVs and furthermore provide a strategy to overcome ARV function in the clinic.

Introduction

AR contains an N-terminal transactivation domain (encoded by exon 1), the DNA binding domain (exons 2-3), a short hinge region (exon 4), and the C-terminal ligand binding domain (LBD; exons 4-8) where the androgenic ligands testosterone and dihydrotestosterone bind [1]. When bound by androgens, AR undergoes a conformational change that permits nuclear translocation, DNA binding and regulation of AR target genes [2]. AR signaling is required for development of the normal prostate [3] and for prostate cancer progression, even in the end stage of castration-resistant disease [4].

Previous studies have identified up to 7 different ARVs, all isolated from the CWR22R system [5], which share the common structural feature of an N-terminus encoded by exons 1/2 or exons 1/2/3 of AR followed by variable C-terminal sequences (cryptic exons) originating from introns 2 or 3 [6-8]. All these ARVs lack the LBD and are purported to have constitutive, ligand-independent activity. Using isoform-specific RTPCR, one variant designated AR-V7 [7] or AR3 [8] (hereafter called AR-V7) has been detected in other human prostate cancer cell lines and xenografts as well as normal and malignant human prostate tissue samples. AR-V7 levels are generally higher in castration-resistant versus androgen-dependent tumors, and AR-V7 expression in early stage prostate cancer has been associated with a worse prognosis following radical prostatectomy. Based on evidence that AR-V7 can enhance growth of androgen-dependent xenografts in castrated mice [8], it has been proposed that ARVs can function as drivers of castration resistance [6-8]. Here we demonstrate a greater diversity of ARVs than previously appreciated which have different activities, ranging from constitutively active to

dominant negative. In addition, we show that ARV expression is negatively repressed by androgen. Some ARVs promote castration resistance or anchorage-independent growth, but they do so by acting through AR-FL and not independently. These findings have implications for whether and how ARVs cause castration resistance and for strategies to overcome their gain-of-function properties.

Material and Methods

ARV discovery: AR 3′ RACE PCR was performed using mRNA isolated from tumors growing in castrated mice; spontaneous castrate resistant Myc-CaP and 14d post-castration for VCaP tumors.

Next-generation sequence analysis: 454 reads were processed using the TopHat alignment algorithm [26] to identify splice junctions. Logarithmic SOLiD read coverage was mapped to the AR locus, except for reads spanning splice junctions.

Plasmids and cell transduction: cDNA for mouse AR isoforms and human ARVs were cloned from Myc-CaP and 22Rv1, respectively, into Retro-X Q vectors (Clontech) as was EGFP. human AR-FL in pWZL/AR [27], was provided by William Hahn. AR or GFP expressing stable cell lines were derived after pantropic retroviral infection (Clontech).

Reagents: The total AR primary antibody used in these studies was raised against an N-terminal epitope (Santa Cruz, N-20). AR-V7 specific antibody was kindly provided by Jun Luo. MDV3100 was synthesized at MSKCC and R1881 was from PerkinElmer.

AR reporter assay: Cells were co-transfected in charcoal-stripped serum with 4X ARE-Luciferase and pRL-TK (Promega) at 10:1. Firefly activity was normalized to Renilla using Dual Luciferase Assay reagent (Promega).

Anchorage independent growth: 10^4 DU145 or 10^5 LNCaP cells were suspended in soft agar with 20% FBS and 10 μM MDV3100 in 0.1% DMSO vehicle. At 2-3 wk, colonies were stained with 0.5% crystal violet, imaged and counted using GelCount (Oxford Optronix).

Tumorigenesis assays: Human cells were injected s.c. into the flank of intact or castrated male CB17 SCID mice (Taconic), while Myc-CaP were grafted into the mammary fat pad of intact male FVB mice (Taconic). Tumor (~500-1000 mm^3) bearing mice were treated by castration or oral 10 mg/kg MDV3100. Testosterone pellets were 12.5 mg/90d release (Innovative Research of America). All animal experiments were performed in compliance with the guidelines of the Research Animal Resource Center of the Memorial Sloan-Kettering Cancer Center.

Results

Identification of structurally diverse ARVs in human and murine prostate cancer models

The family of nuclear hormone receptors undergo extensive splicing that yields multiple, functionally diverse variants [9, 10]. To determine if similar diversity exists for AR, we selected two other prostate cancer cell lines for analysis, the human cell line VCaP [11] and the murine cell line Myc-CaP [12], because we routinely detected lower molecular weight AR protein isoforms (ranging from ~60-80 kDa versus 110 kDa for ARFL) in lysates from these models. To

identify the molecular structure of these smaller isoforms, we performed 3′ RACE followed by standard subcloning and Sanger sequencing and additionally, in the case of VCaP, by next generation RNA-Seq.

Sequencing of AR mRNA transcripts in VCaP, primed from the border of exons 2/3 to focus on annotation of novel 3′ ends, identified the known ARVs AR-V1 and ARV7 (7) as expected as well as 4 novel isoforms (numbered AR-V8 thru AR-V11, following the nomenclature of Hu et al.) (Fig. 4-1A, Table 4-1). The AR isoforms were detected by next generation sequencing platforms (454 and SOLiD) as well as by conventional subcloning and Sanger sequencing of 3′ RACE products. Like AR-V1 and AR-V7, the novel C-terminal sequences of the four new isoforms were derived from AR intron 3. Having validated our RNA-Seq strategy, we considered the possibility of additional ARVs downstream from AR exon 3. This analysis, again confirmed by 454 and SOLiD, revealed evidence of exon skipping (4/6, 4/7, 4/8, 6/8), and an occult exon within intron 5 (in red) (Fig. 4-1B). These data reveal greater complexity of ARVs than previously appreciated, although cloning of complete cDNAs is needed to fully characterize the molecular anatomy of these novel variants. Of note, an exon skipping 4/8 variant was also isolated independently from another xenograft model (called AR^{v567es}) while this work was under review [13].

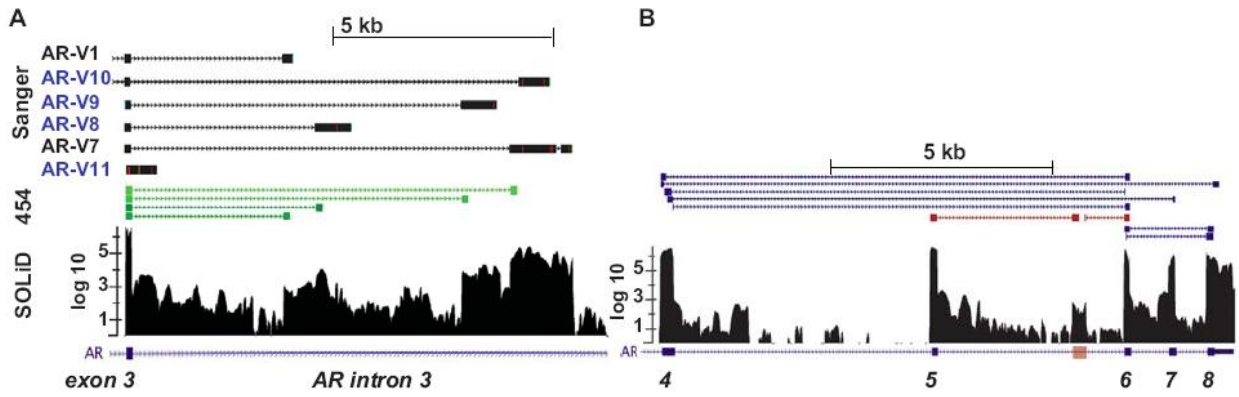

Figure 4-1. Discovery of ARVs in additional prostate cancer models. (A, B) Next generation AR mRNA sequencing in VCaP. A, Known (black) and novel (blue) exon 3 truncation ARVs were initially identified by Sanger sequencing. 454 junctions supporting Sanger sequences were determined by TopHat in supervised mode (i.e. input of predetermined junctions). TopHat does not detect non-canonical splice sites (AR-V10) or exon runon (AR-V11). SOLiD coverage is represented on a log scale. The greatest number of SOLiD reads mapped to the native AR exons, consistent with the relative abundance of AR-FL in these cells. SOLiD specifically detected the unique AR-V11 sequence that was not identifiable using TopHat. B, Unsupervised TopHat analysis of 454 junctions identifies putative ARVs distinct from exon 3 truncations involving exon skipping. A putative cryptic intron 5 exon is shown in red, with a large number of SOLiD reads relative to adjacent intron sequence.

Table 4-1: ARV variants of exon 3 truncation class in VCaP tumor xenografts identified by Sanger and next-gen sequencing.

Variant	Terminal amino acids of exon 3	Distance into intron 3 of unique sequence	C-terminal unique peptide sequence before in-frame stop codon
AR-V1*	MTLG	3430	AAVVVSERILRVFGVSEWLP
AR-V7*	MTLG	8545	EKFRVGNCKHLKMTRP
AR-V8	MTLG	4155	GFDNLCELSS
AR-V9	MTLG	7433	DNLPEQAAFWRHLHIFWDHVVKK
AR-V10	MT	8753	PSSGTNSVFLPHRDVVRTGCRSNSGYHSCSCEYHDYCFL
AR-V11	MTLG	0	GKILFFLFLLLPLSPFSLIF

* Previously described, see text.

In murine Myc-Cap cells, we recovered four novel ARVs which we labeled mARV1-4 (Fig. 4-1C, Table 4-2). Only mAR-V2 and mAR-V4 were pursued further because isoform-specific knockdown experiments validated that these two isoforms encode the most abundant truncated ARVs (Fig. 4-1D). Structurally, mAR-V2 resembles the human isoforms AR-V1 and AR-V7 with retention of AR exons 1-3 but differs with the new C-terminus containing only a single additional amino acid. mAR-V4 is distinct because exons 1-4 are retained prior to addition of novel C-terminal sequence. mAR-V4 structurally is analogous to the recently described human AR^{v567es} [13]. Unlike the human ARVs, the novel C-terminal sequences of mAR-V2 and mAR-V4 are not derived from murine AR introns. Rather, these sequences map outside the murine AR gene to distal regions on the X chromosome (Fig. 4-1C). We do not know the structural basis for the generation of these isoforms at the genomic level, but the fact that Myc-CaP cells have AR

gene amplification (also seen in ~30 percent of castration-resistant human prostate cancer) raises the possibility of intrachromosomal gene rearrangements.

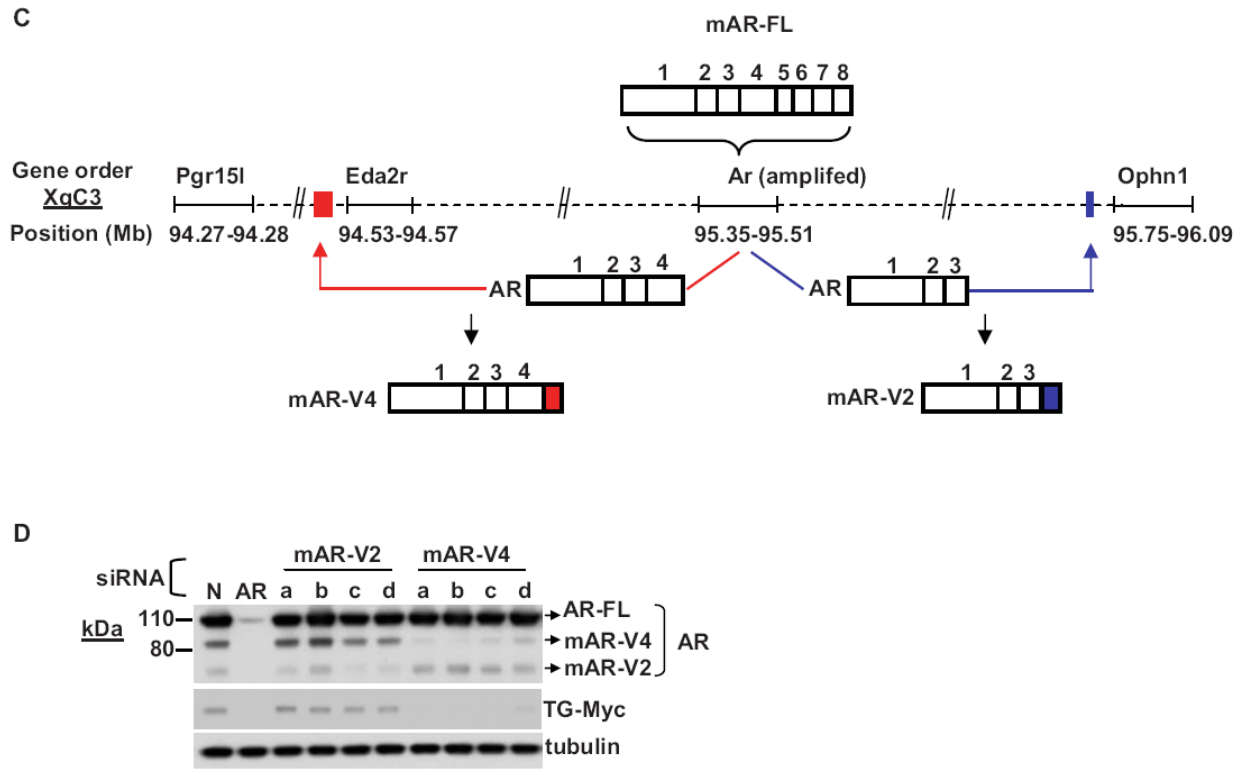

Fig 4-1. Discovery of ARVs in additional prostate cancer models. (C) Sequences of Myc-CaP ARVs were mapped to intergenic regions (dashed lines) of chromosome X (not drawn to scale) using the UCSC Genome Browser (NCBI37/mm9 assembly). The genetic origins of mAR-V2 and mAR-V4 specific sequences are represented by blue or red boxes, respectively. Adjacent genes are shown with their physical position. The ARV and AR-FL proteins are depicted with the native AR exons numbered (not drawn to scale). (D) Myc-CaP were transfected with siRNA against total AR (exons 1 and 3), mAR-V2, or mAR-V4 (4 individual siRNA per ARV, denoted a-d). A non-target siRNA (N) was used as a negative control. Growth media was standard 10% FBS. Western blots were done at 24h post-transfection.

Table 4-2: ARV variants in Myc-CaP tumors identified by Sanger

Variant	Exon composition	Source of unique sequence	C-terminal unique peptide sequence before in-frame stop codon
mAR-V1	1-2-3-Unique	AR intron 3; position 0	GMIFFSSSLLVLLLLTFILYNHCIRSSFSSVILLRVPTRQ
mAR-V2	1-2-3-Unique	intergenic; Ar and Ophn1	D
mAR-V3	1-2-4-Unique	intergenic; Pgr15l and Eda2r	EFQHAVVPHFQDGDAPVSSKAQIKRH
mAR-V4	1-2-3-4-Unique	intergenic; Pgr15l and Eda2r	EFQHAVVPHFQDGDAPVSSKAQIKRH

ARV expression is correlated with AR-FL mRNA levels and induced by castration

Analysis of AR-V7 expression across a range of human prostate tissues has shown low levels in normal prostate and higher levels in castration-resistant prostate cancer samples. In both settings, AR-V7 transcript levels are substantially lower than those of AR-FL. The increase in ARV expression observed with advanced disease could occur through selection of more malignant or castration-resistant subclones or due to hormone-dependent regulation of ARV expression. To distinguish between these possibilities, we examined the kinetics of ARV expression in the VCaP and LuCaP35 human prostate cancer xenograft models, both of which are known to express ARVs.

No ARV protein expression (using an N-terminal AR antibody) was detected in VCaP tumors grown in intact mice. However, a substantial increase in both ARV and AR-FL expression was detected just 2 days post-castration, reaching a maximum at 14 days (Fig. 4-2A,B). Remarkably,

this increase was completely extinguished within 8 days of testosterone replacement. In contrast to VCaP, LuCaP35 xenografts expressed ARVs when grown in intact mice. Castration modestly increased AR-FL and ARV mRNA, but these increases did not result in obviously higher protein levels. As with VCaP, testosterone replacement downregulated both AR-FL and ARV mRNA levels. Whereas ARV protein was no longer detectable, AR-FL protein levels were mostly unchanged, consistent with the well established stabilization of AR-FL by androgen. In retrospect, these data are consistent with older literature documenting androgen-mediated negative feedback of AR-FL expression but now extended to ARV expression [14-17]. The multiple ARV protein profile observed in VCaP tumors is also consistent with the complexity of ARV mRNAs detected by RNA-Seq.

Quantitative analysis of AR-V1 and AR-V7 mRNA levels relative to AR-FL in VCaP and LuCaP35 revealed that ARV levels range from 0.1-1.0 percent of AR-FL mRNA levels (Fig. 4-2B). To extend the analysis beyond xenografts, we addressed the same question in 10 clinical samples from patients with metastatic prostate cancer and found similar ARV/AR-FL ratios ranging from 0.1-2.5 percent (Fig 4-2C). Taken together, these data indicate that ARV expression is hormone-dependent (androgen-repressed), is generally linked to AR-FL levels and is likely to be an acute response to castration therapy rather than a driver of castration-resistant clonal expansion.

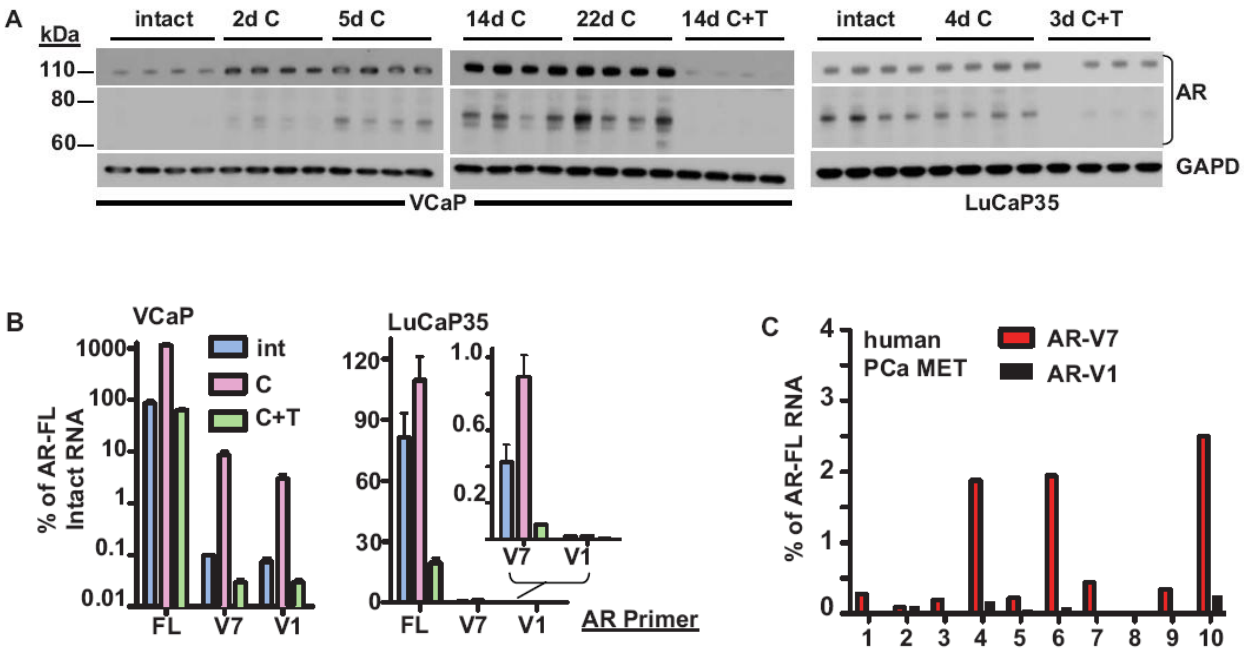

Fig 4-2. Androgen represses AR-FL and ARV transcription. (A) Western blot of prostate cancer xenograft tumors from intact mice or various days post-**C**astration. **T**estosterone was implanted after the indicated number of days post-castration. Duration of testosterone replacement was 8d or 4d for VCaP and LuCaP35, respectively. AR-FL blots underwent extensive additional washing to avoid ECL signal saturation, therefore the actual large AR-FL to ARV protein ratio is not reflected here. (B, C) qRT-PCR of AR isoforms with normalization to β-actin. *B*, Mean expression in xenografts (n=4), error bars sem. 14d post-castration tumors were used for VCaP. *C*, Expression in individual clinical prostate cancer metastases.

Structurally similar ARVs have distinct biological activity

Prior work has suggested that all ARVs lacking the LBD are ligand-independent, constitutively active isoforms. However, the ARV-specific siRNA experiments conducted in Myc-CaP cells under standard growth conditions of 10% FBS (Fig. 4-1D) revealed that expression of the AR-dependent probasin-Myc transgene was abolished by knockdown of mAR-V4 but not of mAR-

V2. qRT-PCR confirmed that this decline in Myc expression was due to loss of probasin-Myc mRNA rather than endogenous mouse Myc (Fig. 4-3). Therefore, the low, basal level of androgen present in 10% FBS was not capable of maintaining probasin-Myc expression after loss of mAR-V4, despite abundant levels of AR-FL (Fig. 4-1D). However, exogenous androgen supplementation restored AR-FL mediated regulation of probasin-Myc, without a requirement for mAR-V4 (Fig. 4-4, day 3 lanes).

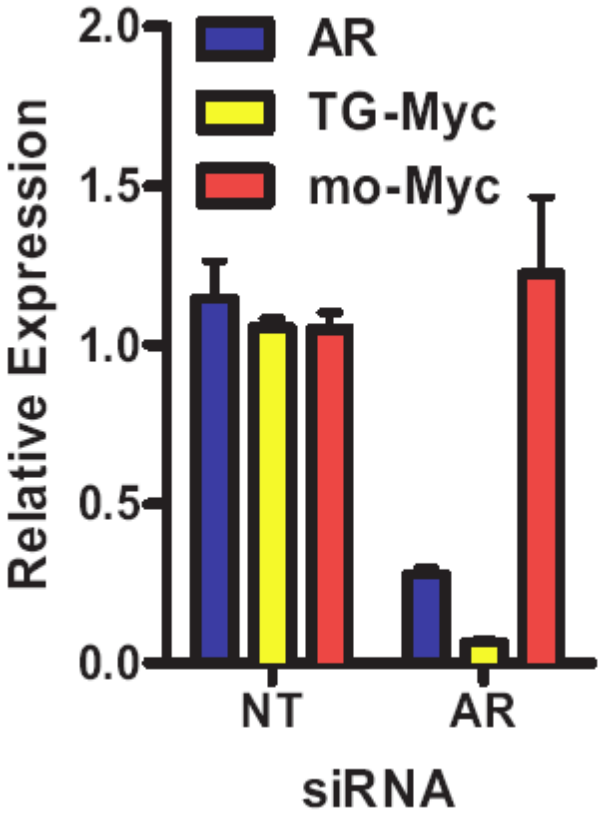

Fig 4-3. AR knockdown results in downregulation of the probasin-Myc transgene mRNA but not the endogenous mouse Myc. Myc-CaP were transfected (n=3) with 100 nM non-target (NT) or AR siRNA. Day 1 posttransfection, AR, transgene human Myc (TG-Myc), and mouse Myc (mo-Myc) mRNA levels were quantified by qRT-PCR. error bars sem.

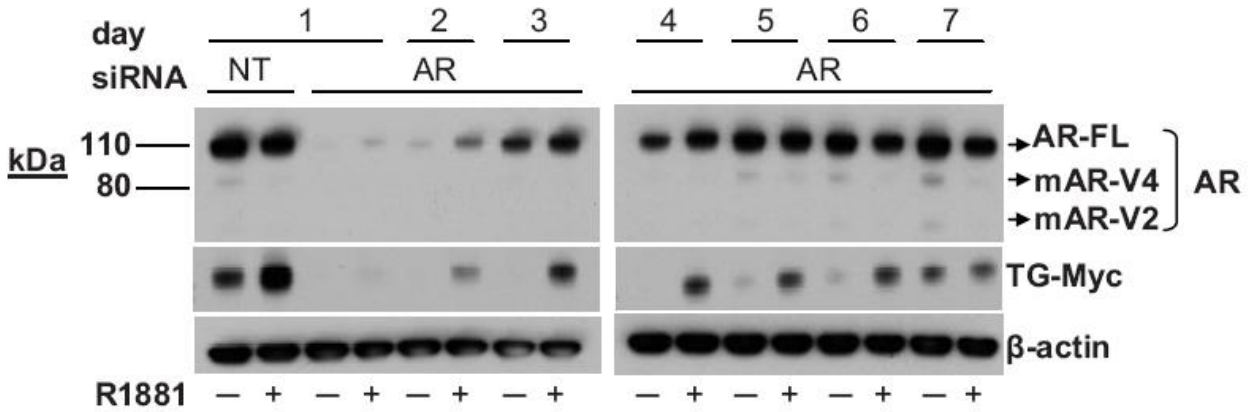

Fig 4-4. Probasin-Myc transgene is regulated in either a ligand-dependent (AR-FL) or ligand-independent (mAR-V4) manner. Myc-CaP were transfected with 100 nM non-target (NT) or AR siRNA in the presence or absence of 1 nM R1881 in 10% charcoal stripped serum. The transient nature of siRNA effects were exploited to monitor recovery of AR-FL and mAR-V4 expression after knockdown over time by Western blot for each of days 1-7 post-siRNA transfection. Expression of probasin Myc (TG-Myc) was also assessed for each day.

The fact that mAR-V2 and mAR-V4 differed in their ability to regulate probasin-Myc suggests individual ARVs may not be functionally equivalent despite similar structural features. Therefore, we directly compared the biological function of three ARVs truncated after exon 3 (AR-V1, AR-V7 and mAR-V2) that differ only in the amino acid sequence encoded by the C-terminal extension and a fourth ARV (mAR-V4) which represents the new class of ARVs with truncation after exon 4 (Fig. 4-5A). These four ARVs are also the most abundantly expressed alleles in the human and murine models studied to date. Following transient transfection of expression plasmids into Cos-7 cells, all four ARVs were robustly expressed at levels equal to or higher than AR-FL (Fig. 4-5B) but they differed in their subcellular localization. AR-V7 and

mAR-V4 were nuclear whereas AR-V1 and mAR-V2 were cytoplasmic (Fig. 4-5*C*). We next measured their transcriptional activity in prostate cancer cells, choosing the AR-negative DU145 cell line to avoid potential confounding effects of endogenous AR-FL. Using the AR-dependent luciferase reporter vector (4X ARE-Luc) as a readout, the activities of AR-V7 and mARV4 were comparable to hormone-induced AR-FL activity whereas AR-V1 and mAR-V2 were only modestly above background (Fig. 4-5D). These data establish that some, but not all, ARVs can function in isolation (without AR-FL) to activate AR reporter constructs and that this correlates with nuclear localization.

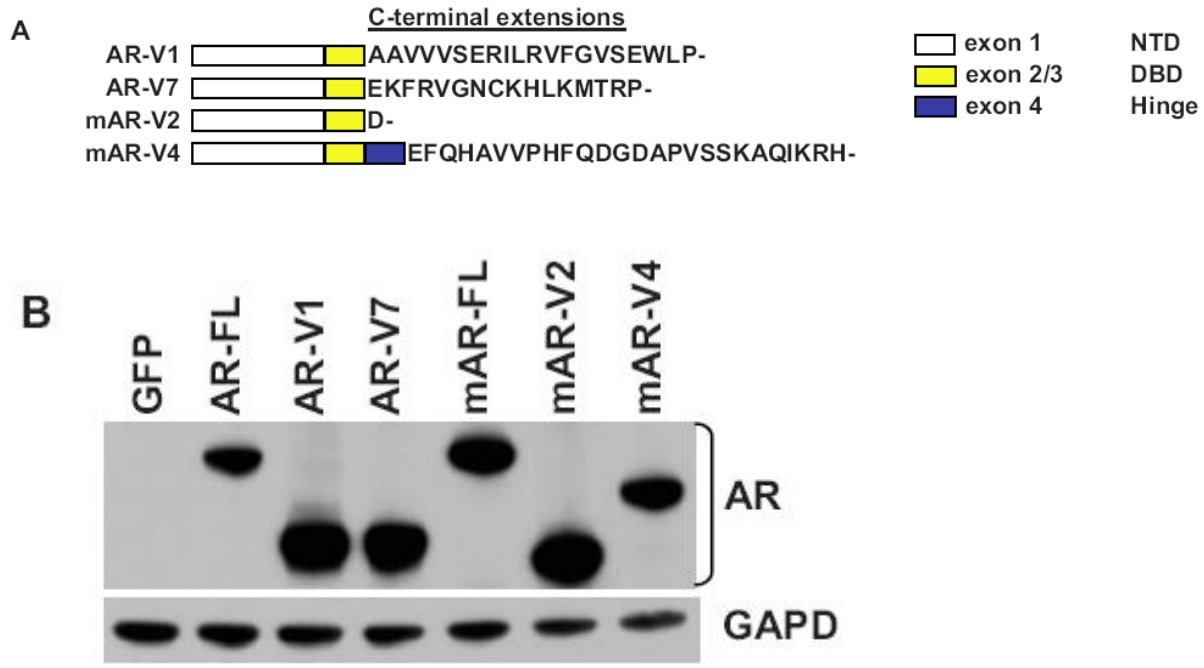

Fig 4-5. LBD truncation is insufficient for nuclear translocation and androgen-independent transcriptional activity. A, Peptide sequences of the C-terminal extension for each ARV used in comparative studies. B, Western blot of transiently transfected Cos-7.

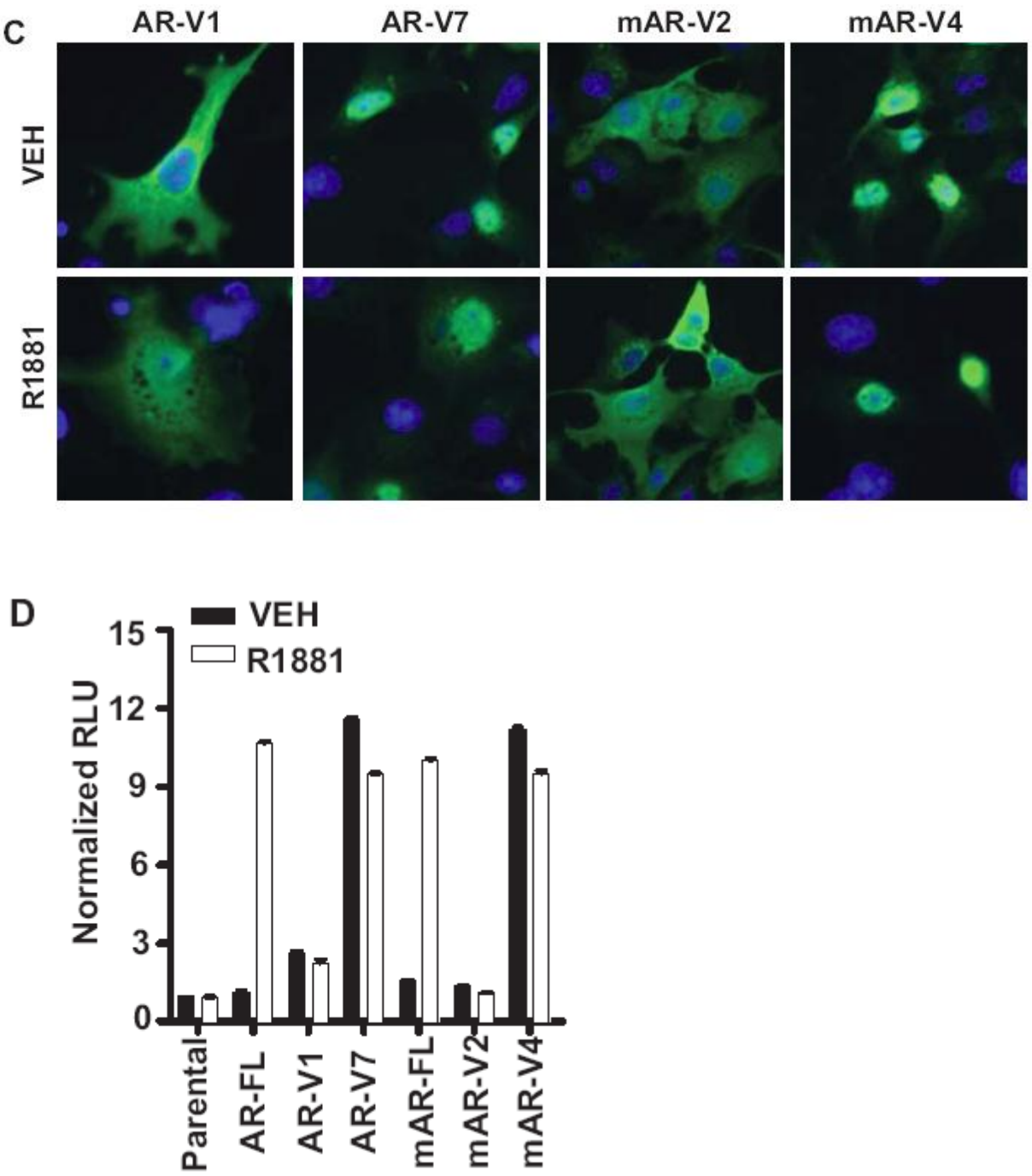

Fig 4-5 (C, D) C, AR immunofluorescence +/- 1 nM R1881 of transiently transfected Cos-7 cells; 200X. D, Representative transfected 4X ARE-Luc activity normalized to Renilla-Luc.

Curiously the level of ARV protein expression, particularly for the constitutively nuclear isoforms AR-V7 and mAR-V4, was reduced relative to AR-FL in stable lines despite equal or greater levels of ARV mRNA expression (Fig. 4-6A). This pattern was seen in DU145 (Fig. 4-6B), LNCaP (Fig. 4-6C), and Myc-CaP (Fig. 4-6D) cells stably transduced with ARVs. Treatment with the proteasome inhibitor MG132 increased the level of some ARVs, providing some evidence that ARVs may be less stable (Fig. 4-6E). However, some but not all tumors derived from LNCaP sublines expressed abundant levels of ARV protein (Fig 5*B*), suggesting other variables may be at play. Further work is required to understand the complex relationship of ARV mRNA and protein levels. Serendipitously, these stable lines reflect the relative AR-FL/ARV ratios seen naturally in human tumors and xenografts (Fig. 4-2).

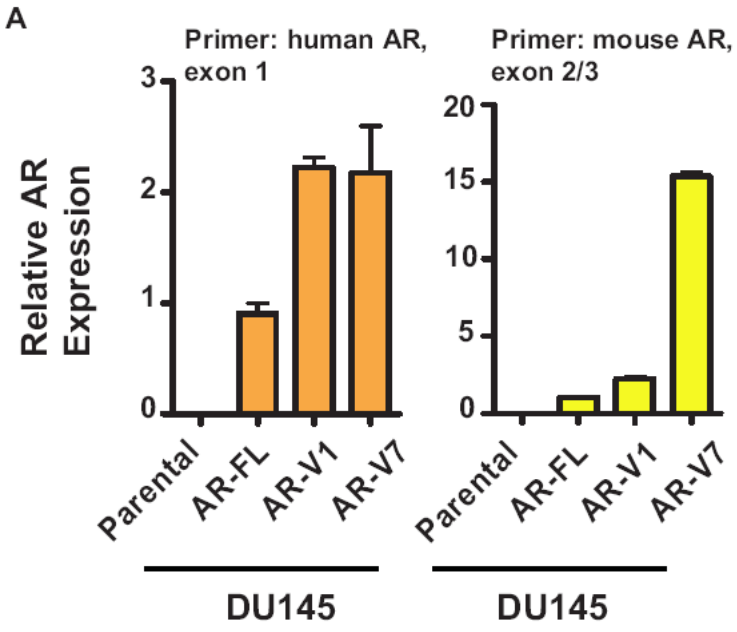

Fig 4-6 ARV mRNA levels and protein do not consistently correlate. A, Steady-state levels of AR mRNA determined by qRT-PCR in stable lines of DU145 (n=2) expressing individual human (left) or mouse (right) AR isoforms. error bars sem.

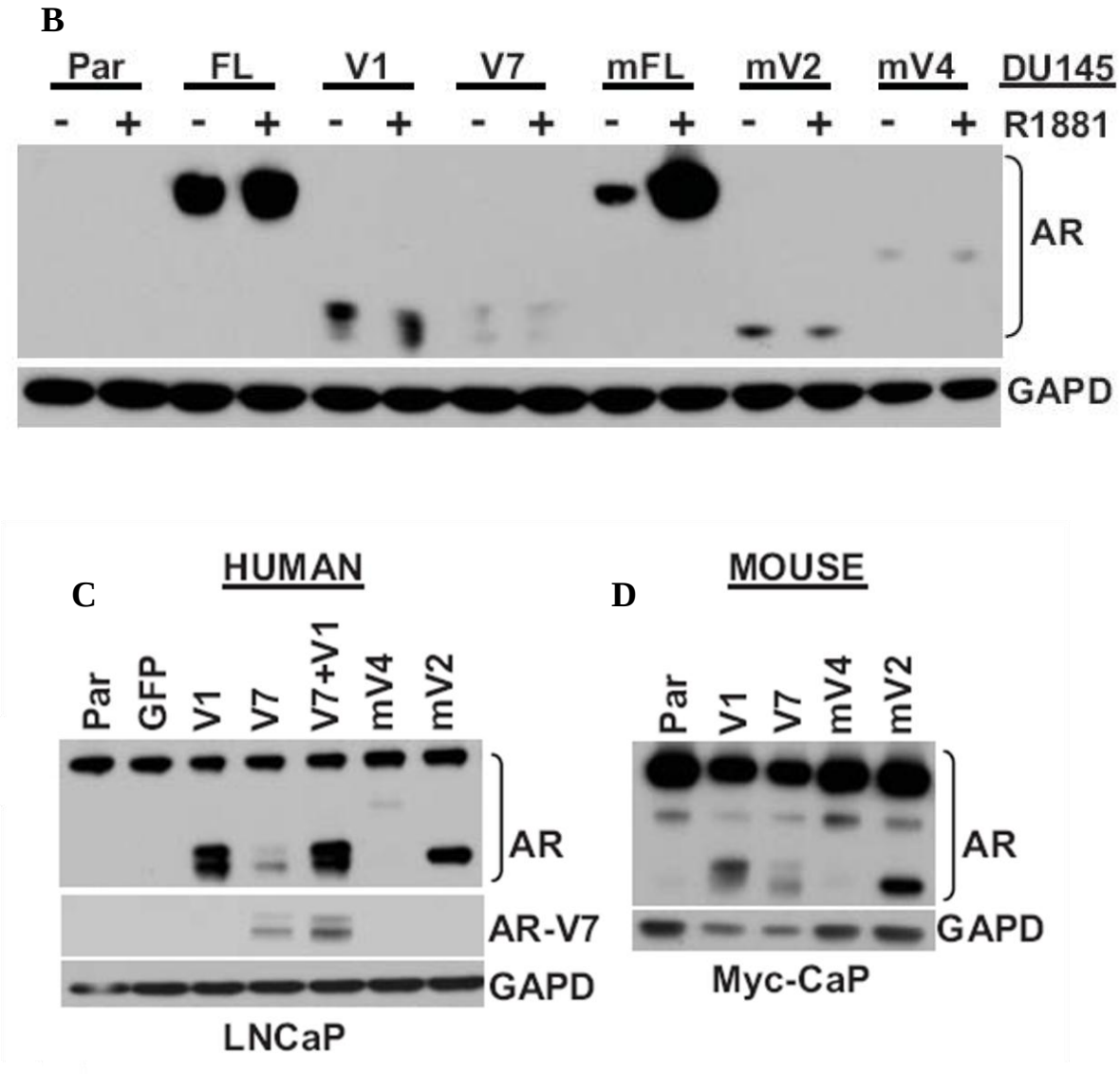

Fig 4-6 (B, C) Western blot of stably infected DU145 (B), LNCaP (C) and Myc-CaP (D) prostate cancer cell lines.

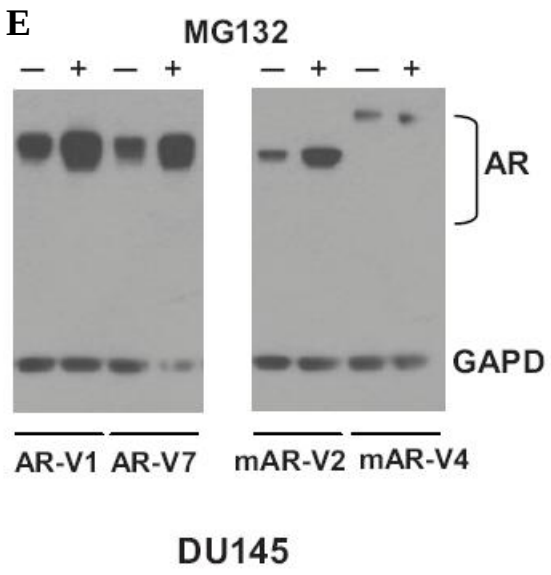

Fig 4-6E Western blot of DU145-AR cells treated with 10 μM MG132 or vehicle control for 6hr.

We next compared the impact of the four ARVs on castration-resistant growth in two androgen-dependent xenograft models, LNCaP (human) and Myc-CaP (murine). AR-FL expressing models were intentionally selected to recapitulate the clinical scenario of combined AR-FL and ARV expression. After implantation in pre-castrated mice, the AR-V7 and mAR-V4 LNCaP sublines formed larger tumors than parental cells whereas AR-V1 and mAR-V2 had no discernable effect (Fig. 4-7A). In the Myc-CaP model, where tumors in intact male mice consistently regress with castration [12], only mAR-V4 conferred gain-of-function by accelerating the rate of castration-resistant growth (Fig. 4-7B). In summary, only the ARVs with constitutive nuclear localization (AR-V7 and mARV4) displayed ligand-independent biological activity in transcriptional and castration-resistant growth assays. Of these two ARVs, mAR-V4 may be the more potent allele but we cannot exclude potential cross-species differences.

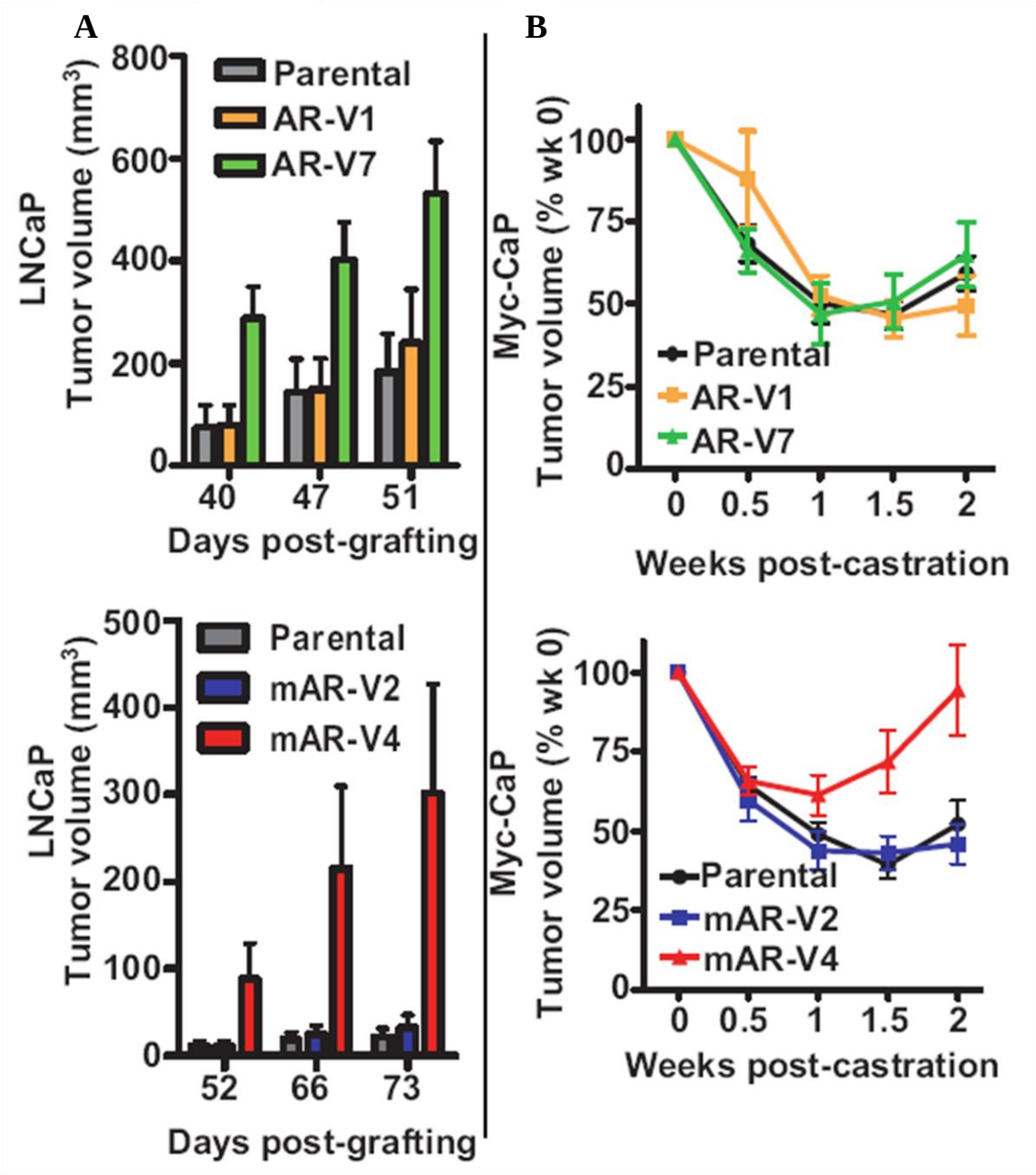

Fig 4-7 Effect of ARVs on prostate cancer tumor growth in castrated mice. A, Parental or stable LNCaP cells were bilaterally grafted into pre-castrated mice (n=6-10). Mean tumor volumes are graphed; error bars sem. The human and mouse ARV LNCaP lines were grafted on separate days. Tumor volumes varied between experiments due to the inherent *in vivo* variability of

LNCaP. B, Parental or stable Myc-CaP cells were bilaterally grafted into intact mice (n=4-8). Castrate-resistant growth was assessed after castration-induced tumor regression. Mean tumor volume is depicted as the percentage of the tumor volume at the time of castration; error bars sem.

In an independent set of experiments, we examined the expression of ARVs in parental Myc-CaP tumors that had naturally progressed to castration resistance after initial growth in intact male mice followed by castration, or after prolonged latency in pre-castrated mice. This analysis revealed that some, but not all, castration-resistant tumors expressed ARVs including mAR-V4. Therefore, high expression of mAR-V4 (and consequently probasin-Myc) was not required for Myc-CaP tumors to spontaneously progress to castration resistance (Fig. 4-8).

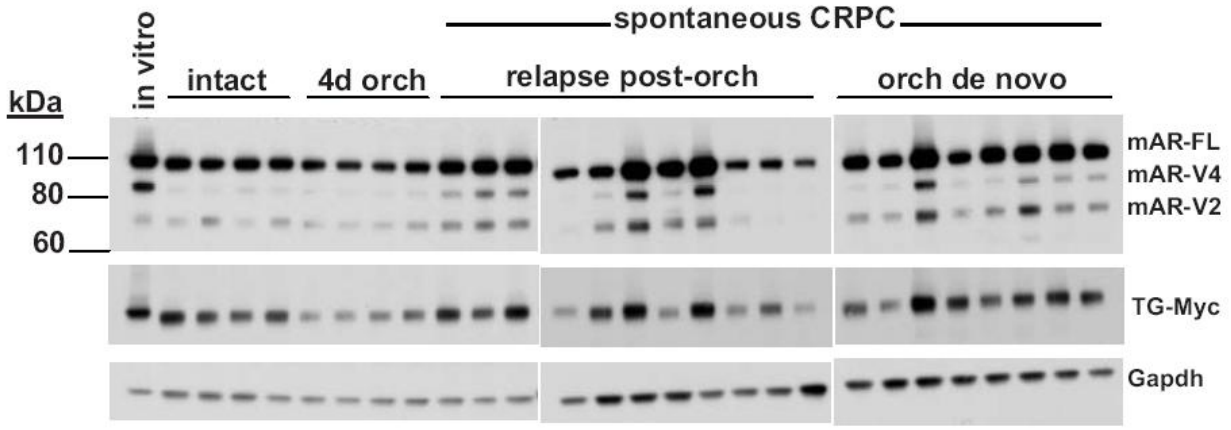

Fig 4-8. Levels of probasin-Myc in Myc-CaP CRPC correlate with mAR-V4 expression, but high probasin-Myc/mAR-V4 are not required for CRPC. Western blots of Myc-CaP tumors growing in intact mice, 4d post-orchiectomy, or spontaneous CRPC that either relapsed after orchiectomy or arose de novo inorchiectomized mice. Myc-CaP lysates harvested from *in vitro* are shown for comparison.

Gain-of-function ARVs require AR-FL

The novel antiandrogen MDV3100 has shown impressive clinical activity in end-stage, castration-resistant prostate cancer and is currently under evaluation in a phase III registration trial [18, 19]. MDV3100 acts on AR-FL by directly binding the LBD; therefore, constitutively active ARVs such as AR-V7 and mAR-V4 could, in theory, confer resistance to MDV3100. We tested this directly in the castration-resistant LNCaP xenograft model which we previously showed is sensitive to MDV3100 [18]. Remarkably, MDV3100 blocked the growth of both GFP and AR-V7-expressing LNCaP xenografts equivalently (Fig. 4-9A). The sensitivity of LNCaP/AR-V7 tumors cannot be explained by loss of AR-V7 expression since moderate to high AR-V7 expression was present in all but one of the MDV3100 treated LNCaP/AR-V7 tumors

(Fig. 4-9B). To explore this unexpected effect of MDV3100 treatment in a more controlled experimental setting, we turned to a highly quantitative soft agar assay. Consistent with the transcriptional output and xenograft studies, only the constitutively nuclear ARVs (AR-V7 and mAR-V4) conferred increased anchorage-independent growth (Fig. 4-9C, D). Interestingly, co-expression of AR-V1 with AR-V7, which is observed in human xenografts and tumors, completely abrogated the gain-of-function conferred by AR-V7, indicating that AR-V1 is likely a dominant negative variant. AR-V7 inhibition by AR-V1 was not due to loss of AR-V7 expression in the double variant line (Fig. 4-6C). As in the xenograft assay, MDV3100 completely blocked the gain-of-function conferred by AR-V7 and mAR-V4. The growth suppression by MDV3100 in agar is specific because colony formation by AR-negative DU145 cells was unaffected.

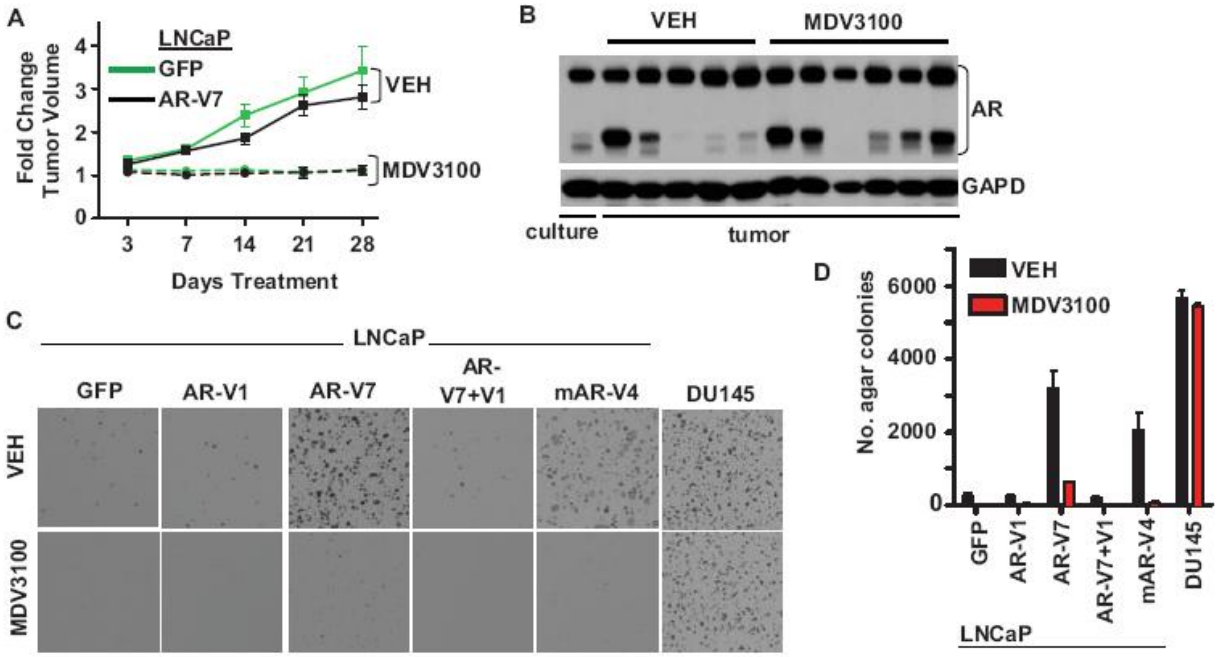

Fig 4-9 Gain-of-function ARVs are not resistant to the antiandrogen MDV3100. A, Pre-castrated mice were grafted with LNCaP/GFP or AR-V7 sublines (n=6-7). When castrate resistant tumors arose, the mice were treated with MDV3100. Fold change tumor volume after treatment is plotted relative to the volume on day 0 of treatment; error bars sem. B, Western blot of LNCaP/AR-V7 cell line (*in vitro* culture) or tumors from castrated mice treated for 25d with MDV3100 or vehicle. (C, D) Anchorage independent soft agar growth in 10 μM MDV3100 for parental DU145 and stable LNCaP lines. C, Images of representative plate quartile. D, Mean total colony number (n=3); error bars sem.

The ability of MDV3100 to reverse the growth advantage conferred by AR-V7 and mAR-V4 is unexpected because both ARVs lack the MDV3100 binding site. To confirm that MDV3100 cannot directly inhibit either allele, we repeated the transcriptional output assays in AR-negative

DU145 cells and saw no effect of MDV3100 on AR-V7 or mAR-V4 but dramatic suppression of AR-FL activity (Fig. 4-10A). Because MDV3100 impairs the nuclear translocation efficiency of AR-FL [18], we considered the possibility of a similar effect on ARVs but saw no evidence that MDV3100 relocalized AR-V7 to the cytoplasm (Fig. 4-10B). In the absence of evidence for a direct effect of MDV3100 on ARVs, we hypothesized that MDV3100 inhibits AR-V7 or mAR-V4 indirectly by targeting AR-FL. Consistent with this model, siRNA directed against the AR C-terminus (designed to specifically target AR-FL) inhibited the growth of LNCaP/AR-V7 and LNCaP/mAR-V4 cells (Fig. 4-11B) to the same degree as control LNCaP/GFP cells. mRNA and protein expression studies confirmed that the C-terminal directed siRNA specifically knocked down AR-FL but not AR-V7 or mAR-V4 (Fig. 4-11A). In addition, expression of the AR-regulated genes TMPRSS2, PSA, and KLK2 were not maintained after selective AR-FL knockdown despite the presence of AR-V7 or mAR-V4 (Fig. 4-11C). We also examined the effect of ARVs on basal expression of three endogenous AR target genes. PSA expression was increased in LNCaP/mAR-V4, consistent with similar findings recently reported for LNCaP cells expressing AR^{v567es} [13]. However, basal PSA expression was decreased in LNCaP/AR-V7 cells (Fig. 4-11D). ARV expression did not alter the basal expression of TMPRSS2 and KLK2 (Fig. 4-11D). These data suggest that ARVs require AR-FL to activate endogenous target genes and that these effects vary in a gene and variant dependent manner.

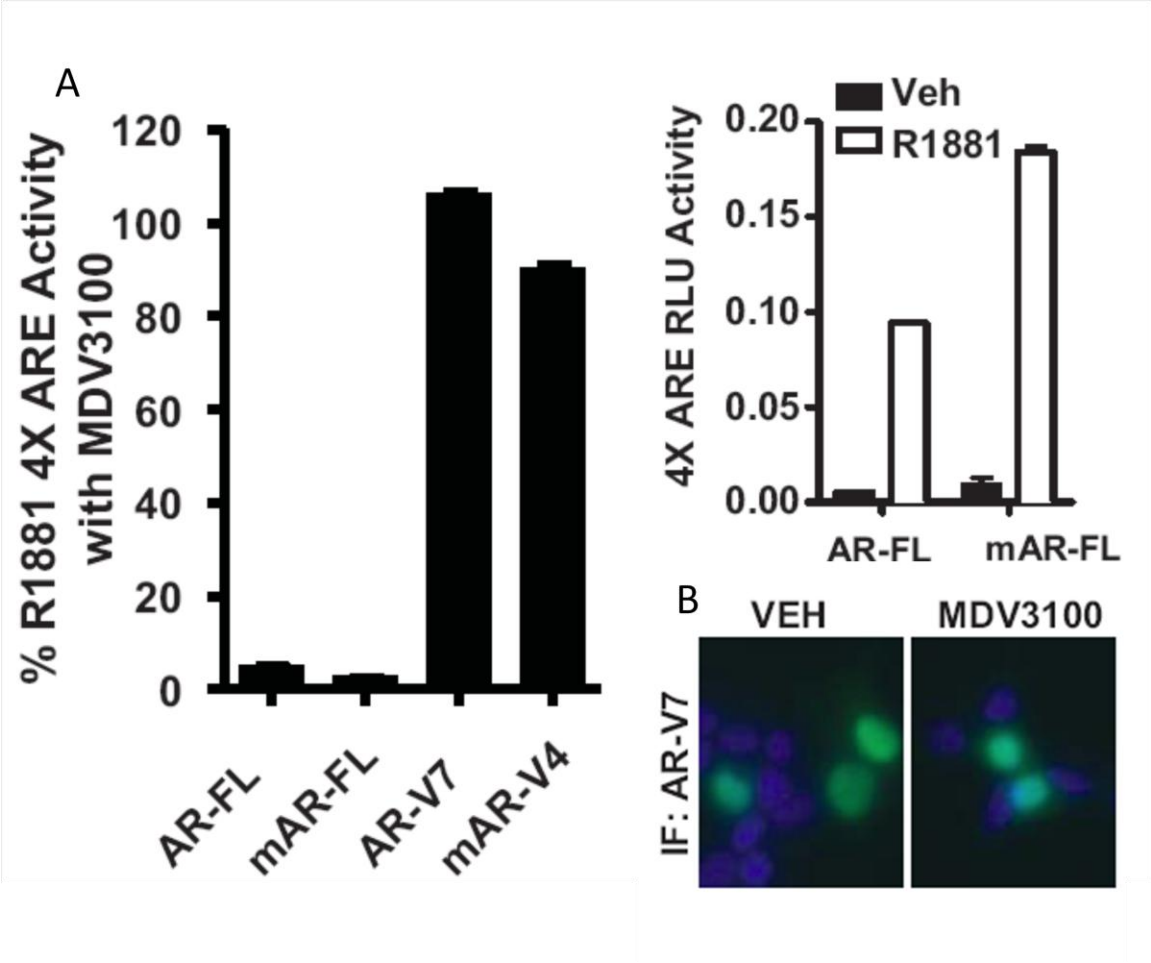

Fig 4-10 MDV3100 does not directly act on ARV transcriptional activity (A) or nuclear localization (B). A, DU145 stable lines with AR-FL (mouse or human), AR-V7, or mAR-V4 were transfected with 4X ARE-Luc and TK-Renilla Luc. Transfected cells were treated with 0.1 nM R1881 and 10 μM MDV3100 for 18 hr. The percentage of R1881-induced 4X ARE-Luc activity remaining with MDV3100 is shown. *inset*- amount of 4X ARE-Luc activity induced by 0.1nM R1881 (without MDV3100) in AR-FL cells. B, AR-V7 immunofluorescence in LNCaP/AR-V7 treated with 10 μM MDV3100; 200X.

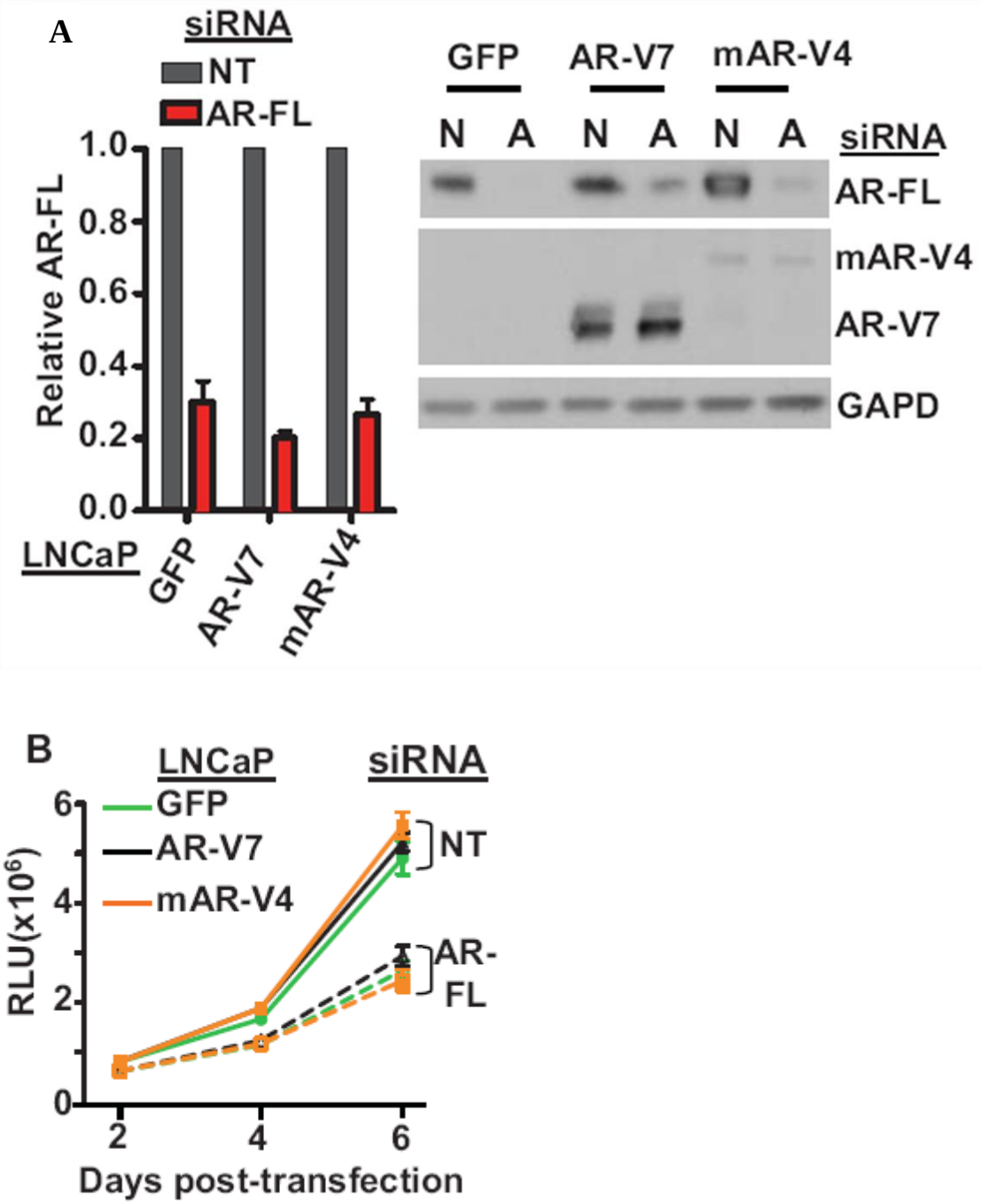

Fig 4-11 Gain-of-function ARVs remain dependent upon AR-FL. A, qRT-PCR for AR-FL in stable LNCaP cells 2d after transfection with 10 nM non-target (NT) or AR-FL siRNA. (n=3); error bars sem. Western blot 6d after transfection with non-target (N) or AR-FL (A) siRNA. B, Proliferation of stable LNCaP cells after transfection with 10 nM NT or AR-FL siRNA.

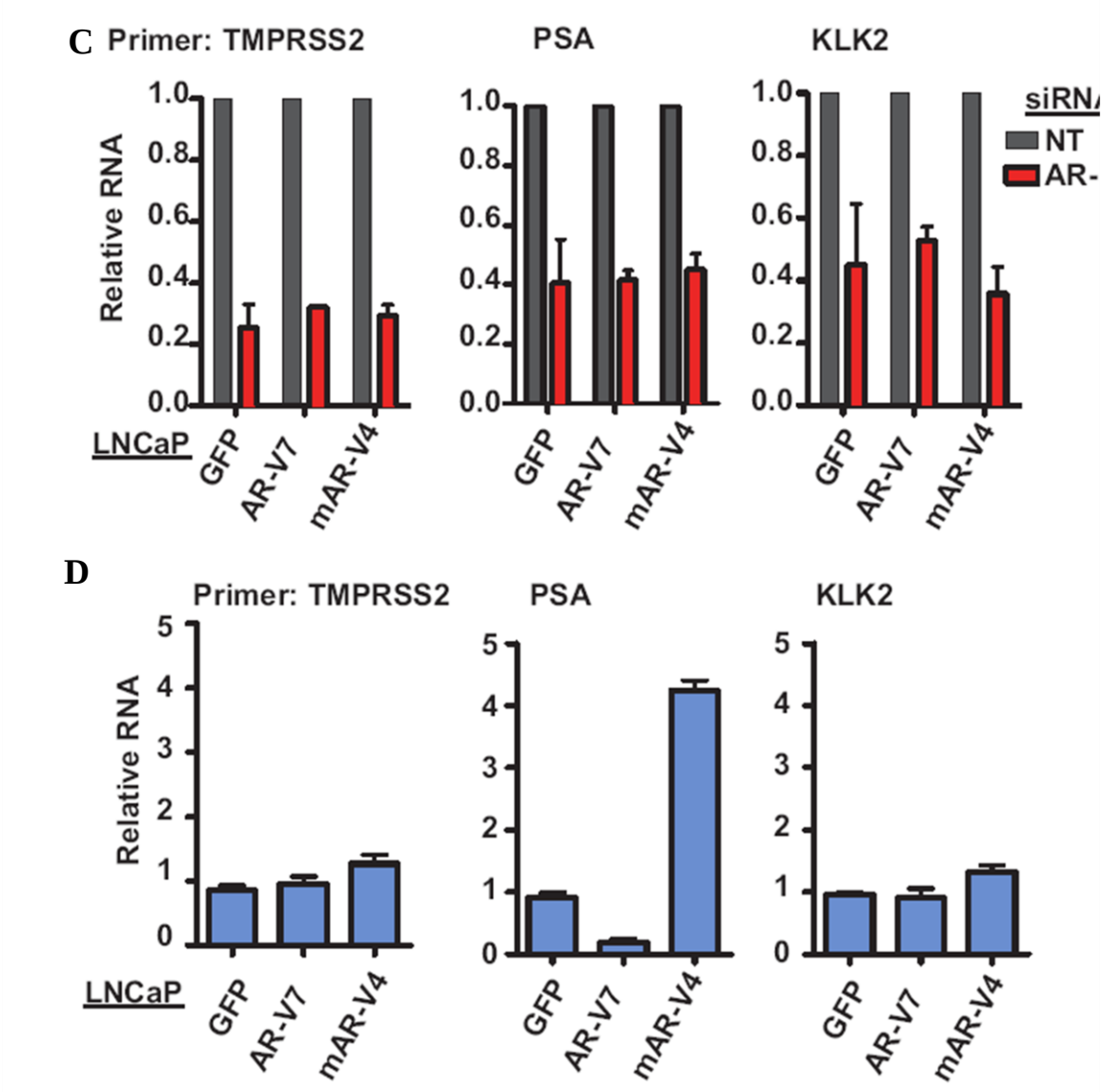

Fig 4-11 (C,D), qRT-PCR for the indicated AR regulated genes 2d after siRNA transfection (n=3); error bars sem. D, Within each LNCaP line, gene expression after AR-FL siRNA is expressed relative to the mean signal of the NT siRNA control, which was set at 1. E, In the NT siRNA controls, the mean expression level of each gene within LNCaP/GFP was used as the reference sample for comparison to the corresponding expression in the LNCaP/ARV lines. Gene expression was normalized to AR-FL.

Discussion

Earlier reports of ARVs in prostate cancer have suggested a general model whereby these alleles are constitutively active and can substitute for the canonical androgen-dependent AR-FL signaling required for prostate cancer growth. If correct, ARVs could emerge as a primary cause of resistance to castration and/or conventional antiandrogen therapy directed at the LBD. By analyzing a greater range of prostate models, we reveal greater structural diversity of ARVs than previously appreciated as well as highly divergent biologic activity among ARVs that, on the surface, appear structurally similar. Unexpectedly, we find that biologically active ARVs require AR-FL to activate endogenous AR target genes and confer castration-resistant growth.

Currently there is little insight into how ARVs are generated. Our studies comparing ARV and AR-FL levels in several xenografts and patient samples establish that ARVs are expressed at much lower levels (~0.1-2.5%) relative to AR-FL. Furthermore, this ratio is generally maintained when AR-FL transcription is enhanced (by castration) or suppressed (by testosterone treatment). These data suggest that ARVs are likely generated through splicing errors that occur in normal or malignant tissues. Therefore, ARV levels would typically be dependent on AR-FL levels rather than a specific splicing defect that favors ARV production. This model does not rule out that other variables, such as structural alterations in the AR gene (amplification, mutation) may impact interaction of pre-mRNAs with the splicing machinery. Indeed, the atypical structure of the ARVs isolated from Myc-CaP cells could be a consequence of AR gene rearrangements that developed in conjunction with AR gene amplification. Point mutations in the AR gene affecting splice recognition sites might also favor the production of certain ARVs.

By characterizing the biological properties of four different ARVs, we uncovered a correlation between constitutive nuclear localization and ligand-independent activity in transcription readouts and growth assays. The fact that only one of the three ARVs truncated after AR exon 3 was nuclear (AR-V7) establishes the importance of the new C-terminal sequence in defining the biological activity of the resulting ARV. The nuclear localization sequence in AR-FL spans exons 3/4 [20, 21] and is therefore partially deleted in all ARVs with exon 3 truncation. ARVs of the exon 3 class are therefore likely to be cytoplasmic unless the novel C-terminal sequence enables nuclear translocation. Of note, analysis of the C-terminal extension from AR-V7 (PredictNLS Online) did not reveal a predicted nuclear localization signal, so it is not clear how AR-V7 reaches the nucleus. Interestingly, immunostaining of AR-V7 has revealed cytoplasmic localization in many clinical samples, suggesting that additional variables regulate nuclear localization [8]. In contrast, ARVs such as mAR-V4 and AR^{v567es} that are truncated after exon 4 retain a complete nuclear localization signal and should be constitutively nuclear unless additional C-terminal sequence is inhibitory or functions as a cytoplasmic anchor or nuclear export signal.

The fact that nuclear ARVs require AR-FL to confer gain-of-function was unexpected particularly since they are capable of activating AR reporter constructs in AR negative cell lines. The pharmacologic (MDV3100) and genetic (AR-FL specific siRNA knockdown) evidence presented here suggest that ARVs function upstream of AR-FL in models that most closely mimic the clinical scenario of combined AR-FL and ARV expression. Whereas the models

studied here argue for sustained dependence of ARV-expressing castration-resistant prostate tumors on AR-FL (and hence sustained sensitivity to LBD-targeted drugs), it is possible that the combined, independent effects of AR-FL and ARVs are required for growth. Some castration-resistant prostate tumors may express high ARV at the expense of AR-FL, thereby potentially bypassing a requirement for AR-FL.

At a biochemical level, the simplest model to explain the AR-FL dependence is ARV/AR-FL heterodimer formation. Dimerization could occur through interaction of the AR-FL C-terminus with the N-terminal FxxLF motif in AR exon 1 (retained in all ARVs). These domains are already known to mediate intra- or intermolecular AR-FL N/C interactions [22, 23]. ARV/AR-FL complexes have not, however, been detected in the CWR22R model by standard co-immunoprecipitation [8, 24]. However, AR-FL and AR^{v567es} were reported to form heterodimers when both were exogenously introduced into AR-null cells [13]. The much greater abundance of AR-FL relative to ARVs makes detection of complexes challenging, such that ARV-specific antibodies are needed to fully examine whether endogenous complexes are present.

Whatever the mechanism, the functional relationship between ARVs and AR-FL raises questions about the repertoire of AR target genes. One possibility is that ARVs simply substitute for hormone and activate an identical set of AR targets. However, transcriptome analysis after selective AR-FL or AR-V7 knockdown or comparing AR-FL to AR^{v567es} revealed distinct subsets of genes regulated by AR-FL, ARVs or both [8,13]. Even among classic endogenous AR

target genes, our data showed differential expression levels dependent upon the particular gene and ARV present, providing evidence that the biology of ARVs is not wholly synonymous with that of androgen-stimulated AR-FL. This model of additional ARV target genes is consistent with our observation that AR-V7 and mAR-V4 stimulate soft agar growth beyond that observed with AR-FL alone. Since this increase in growth remains dependent on AR-FL, we suspect that ARVs and AR-FL jointly drive growth by regulating a greater repertoire of targets than is regulated by AR-FL alone.

Collectively, the data presented here suggest that the role of ARVs in castration or antiandrogen resistance differs from classic models of drug-resistant alleles. Resistance to kinase inhibitors in diseases such as lung cancer, chronic myeloid leukemia and gastrointestinal stromal tumor is often caused by mutations in the targeted kinase domain, present in a small fraction of tumor cells that gradually emerge under the selective pressure of kinase inhibitor therapy [25]. On the surface, ARVs could function similarly. However, our studies reveal that ARVs are expressed within days of castration and disappear within days of androgen treatment, providing clear evidence of acute hormonal regulation. Therefore, ARV expression is more likely a reaction to hormone therapy rather than a driver of castration or antiandrogen resistance. Furthermore, multiple ARVs (typically AR-V1 and AR-V7) are simultaneously detected in patient tumors. The fact that AR-V1 dominantly inhibits AR-V7 provides further evidence that any role of ARVs in clinical castration resistance is likely to be complex. Careful annotation of ARV expression in prostate cancer patients after initiation of conventional or next generation hormone therapy and at relapse should help clarify these issues. The fact that AR-FL is required for ARV function validates continued efforts to develop even better antiandrogens targeting the LBD.

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