

1 **Convergent evolution of p38/MAPK activation in hormone resistant prostate**  
2 **cancer mediates pro-survival, immune evasive, and metastatic phenotypes**

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24 **Summary (120 words)**

25           Adaptation of cancer cells to targeted therapy follows ecological paradigms  
26 observed in natural populations that encounter resource depletion and changing  
27 environments, including activation of pro-survival mechanisms, migration to new  
28 locations, and escape of predation. We identified the p38 MAPK pathway as a common  
29 molecular driver of these three responses during the adaptation to hormone therapy  
30 resistance in prostate cancer. The p38 pathway is activated in therapy-resistant cells and  
31 mechanistically drives these three convergent responses through sustained AR activity,  
32 enhanced invasion and metastasis, and immune evasion. Targeting p38 signaling may  
33 represent a new therapeutic strategy to treat men with metastatic, hormone therapy-  
34 resistant prostate cancer.

35

36 **Keywords**

37 drug resistance; androgen receptor; enzalutamide; prostate cancer; phenotypic  
38 convergence; convergent evolution; Snail; castration resistance; PD-L1

39

40 **Introduction**

41           Populations of individuals within an ecological niche must acquire the necessary  
42 resources to survive and propagate their genetic material to the next generation.

43 Individuals have adapted a wide range of strategies to ensure resource acquisition in an  
44 ever-changing environment. Some of these strategies include dormancy (Varpe, 2017),  
45 hibernation (Kilduff, 2004), migration, and avoidance of predation (Skov et al., 2010).

46 Cancer cells adapt similar strategies within the context of the ecological niche of  
47 the body to cope with variations in resource availability that promotes their survival. A  
48 major resource for cancer cells is the oncogene signaling pathway to which they are  
49 addicted. For prostate cancer cells, this critical lineage oncogene is most often the  
50 androgen receptor (AR) (Mills, 2014). AR activity is pharmacologically targeted through  
51 blocking the ligand binding domain or inhibition of androgenic ligand biosynthesis.  
52 Enzalutamide, a 2<sup>nd</sup> generation inhibitor of the androgen receptor, and abiraterone  
53 acetate, an androgen synthesis inhibitor, each delay progression and improve the survival  
54 of men with both early and late castration-resistant prostate cancer (Beer et al., 2014; de  
55 Bono et al., 2011; Penson et al., 2016; Ryan et al., 2013; Scher et al., 2012). These potent  
56 hormonal therapies significantly prolong the overall survival of men with metastatic,  
57 castration-resistant prostate cancer; however, acquired resistance to these drugs over a  
58 median of one to two years is inevitable.

59 Upon disease progression with enzalutamide or abiraterone treatment, most  
60 tumors remain AR dependent and have a rise in serum levels of prostate-specific antigen  
61 (PSA) (Bluemn et al., 2017; Bryce et al., 2017). Multiple mechanisms of AR signal  
62 restoration have been identified that directly impact the AR gene, including AR  
63 amplification, AR mutations, genomic structural rearrangements (Li et al., 2011b; Li et  
64 al., 2012; Liu et al., 2013; Ware et al., 2014), and alternative splicing events (Liu et al.,  
65 2014). Additionally, alternative mechanisms can promote the AR transcriptome or  
66 promote AR activity through substitute methods of AR activation (Arora et al., 2013;  
67 Ware et al., 2014) to generate a pro-survival response (Chen et al., 2004; Viswanathan et  
68 al., 2018).

69           In addition to AR activation, complementary pro-survival responses focus on  
70 metabolic plasticity, such as dormancy and hibernation. Cancer cells adapt their energetic  
71 needs to accompany survival and fitness in hostile environments (Lehuede et al., 2016),  
72 and disseminated tumor cells can be found in many prostate cancer patients prior to any  
73 clinical symptoms (Ruppender et al., 2013; van der Toom et al., 2016). Furthermore,  
74 organisms and cancer cells alike must avoid predation to ensure their survival in any  
75 environment. Prostate cancer cells avoid the predation of the immune system in a number  
76 of ways, including 1) secretion of immunosuppressive molecules, such as TGF- $\beta$  (Yang  
77 et al., 2010; Yoshimura and Muto, 2011) and soluble WNT ligands (Robinson et al.,  
78 2015), and 2) expression of cell surface or cellular immune checkpoint molecules  
79 (Antonarakis et al., 2020; Gao et al., 2017; Graff et al., 2016). For example, TGF- $\beta$  has  
80 been identified as a potent immunosuppressive ligand, which can be regulated through  
81 Snail to mediate downregulation of HLA-I and promote immune escape (Chen et al.,  
82 2015). Similarly, the immunosuppressive ligand PD-L1 is upregulated in response to  
83 enzalutamide-resistant progression, both in tumor cells and in circulating immune subsets  
84 (Bishop et al., 2015; Graff et al., 2016). Additionally, we have shown that PD-L1  
85 expression is more prevalent on circulating tumor cells from metastatic prostate cancer  
86 patients who are progressing on abiraterone acetate or enzalutamide treatment (Zhang et  
87 al., 2018).

88           In the present study we sought to understand the adaptations to hormone therapy  
89 resistance in prostate cancer. Using an integrated genomics approach we observed that  
90 enzalutamide-induced AR signaling blockade induces convergent phenotypic evolution  
91 on three ecological responses: 1) altered resource acquisition to promote cellular

92 persistence and survival through oncogene re-activation, 2) upregulation of  
93 migratory/invasive factors, and 3) avoidance of predation by immune evasion and  
94 immune suppression (**Fig. 1**). All three of these phenotypes converge across different  
95 model systems on the p38/MAPK stress response pathway, which is highly activated in  
96 human prostate cancer metastases and can be therapeutically leveraged to simultaneously  
97 target and limit these pro-survival responses to overcome enzalutamide resistant growth  
98 and survival.

99

## 100 **Results**

### 101 **Enzalutamide resistant cells exhibit diverse genomic adaptations**

102 Enzalutamide is a potent inhibitor of AR activity (Tran et al., 2009) that initially  
103 induces a response in most men with metastatic castration resistant prostate cancer;  
104 however, progression on enzalutamide typically develops within 1-2 years (Beer et al.,  
105 2014). A deeper understanding of the mechanisms underlying the evolution of  
106 enzalutamide resistance is needed to target these resistance mechanisms. To identify  
107 common molecular mechanisms of enzalutamide resistance we developed a panel of four  
108 enzalutamide-resistant (enzaR) cell lines, LNCaP-enzaR, CS2-enzaR, LN95-enzaR, and  
109 MDA-PCa-2b-enzaR, by chronic, long-term exposure to increasing doses of  
110 enzalutamide. For LNCaP and MDA-PCa-2b cells, the cells were initially exposed to 1  
111  $\mu\text{M}$  enzalutamide and allowed to grow to confluence. The dose of enzalutamide was  
112 doubled at each subsequent passage until cells were capable of sustained growth in the  
113 presence of 50  $\mu\text{M}$  enzalutamide (**Fig. 2A**), which is above the concentration observed in  
114 men with metastatic, castration resistant prostate cancer (Scher et al., 2010). In parallel

115 with development of these enzalutamide-resistant models, we also created enzalutamide-  
116 resistant populations of LNCaP sublines, CS2 and LN95. Both CS2 and LN95 (Hu et al.,  
117 2012) cells were first adapted to androgen deprivation therapy (ADT) by passaging in  
118 media supplemented with an increasing ratio of androgen-depleted media. Following  
119 evolution of ADT resistance, cells were then adapted to enzalutamide with increasing  
120 doses as described above (**Fig. 2A**). All enzaR cell lines are characterized by a significant  
121 increase in cell growth and a decrease in apoptosis in response to enzalutamide treatment  
122 (**Fig. 2B**).

123 Analysis of these enzaR cells revealed several clinically-relevant genotypes and  
124 phenotypes. All cell lines had persistent AR expression (**Fig. 2C-D**) and did not have  
125 notable morphologic changes or consistent neuroendocrine differentiation  
126 (**Supplementary Fig. S1A**). The enzalutamide-resistant LNCaP model acquires the  
127 F876L mutation in AR that converts enzalutamide into a partial agonist (Balbas et al.,  
128 2013; Korpál et al., 2013) (**Supplemental Fig. S2**), but lack additional novel AR  
129 mutations. The enzaR LNCaP and CS2 cells express full-length AR, but do not produce  
130 the AR splice variant, AR-V7, which is a known enzalutamide-resistance driver  
131 (Antonarakis et al., 2014) (**Fig. 2C-E**). On the other hand, enza-R LN95 cells upregulate  
132 both full length AR and AR-V7 (**Fig. 2C-E**). Enza-R CS2 cells display upregulation of  
133 the glucocorticoid receptor mRNA and genomic loss of *RBI* and *BRCA2*, which are  
134 known drivers of resistant and aggressive prostate cancer (Arora et al., 2013; Chakraborty  
135 et al., 2019). None of these lines have upregulation of biomarkers of neuroendocrine  
136 lineage plasticity as measured by co-*RBI* and *TP53* genomic loss or transcriptional  
137 upregulation of *SOX2*, and none of these lines expresses markers of neuroendocrine

138 lineage plasticity (Mu et al., 2017) at the mRNA level (**Supplemental Fig. S1B**). We also  
139 did not observe changes in the stemness markers *CD133*, *NANOG*, or *OCT4* in the enzaR  
140 cell lines (**Fig. 2E, Supplemental Fig. S3**). Thus, this panel of cell lines parallels the  
141 heterogeneous spectrum of AR-positive prostate cancer phenotypes most commonly  
142 observed in the clinic upon progression on enzalutamide.

143         To further understand the evolution of enzalutamide resistance in these  
144 heterogeneous models of prostate cancer, we analyzed the genome, transcriptome and  
145 phospho-proteome of these paired cell lines prior to enzalutamide exposure and following  
146 adaptation to enzalutamide resistance. Genetically, the enzaR models were all remarkably  
147 unique. While enzaR CS2 and LN95 cells share just one copy number alteration – a gain  
148 in chromosome 20p13-p11.1 – this gain is not present in enzaR LNCaP cells  
149 (**Supplemental Fig. S4**). Likewise, enzaR models acquired relatively few single  
150 nucleotide variants in the exome between paired parental and enzaR cell lines, and of  
151 these alterations, none were shared across all enzaR cells (**Supplemental Fig. S5A-B**).  
152 Together, these data suggest that the enzaR lines do not harbor shared genetic alterations  
153 that contribute to their adaptation to enzalutamide.

154

### 155 **Enzalutamide resistance converges on MAPK signaling and stress response** 156 **pathways**

157         Given the diversity of genetic lesions in the enzaR models, we next sought to  
158 determine the molecular drivers underlying the evolution of enzalutamide resistance.  
159 Across each parental and resistant model system, we performed whole transcriptome  
160 RNA Sequencing to identify consistent changes in RNA expression and pathways

161 associated with resistance. Similar to our DNA-level analyses, we observed relatively  
162 few gene expression changes common to all cell line models (4-5% overlap; **Fig. 3A**).  
163 We also performed a reverse phase protein array (RPPA) and evaluated a subset of  
164 proteins and phosphoproteins that are implicated in cancer progression (Akbani et al.,  
165 2014). The proteomics data revealed phospho-p38, a key signaling node in the cellular  
166 stress response, as the top upregulated phospho-protein in all three enzaR cell lines (**Fig.**  
167 **3C**). Reanalysis of our RNA-Seq data at the pathway level revealed common  
168 transcriptional enrichment of MAPK signaling, DNA repair, and several stress-response  
169 pathways (**Fig. 3B**). Importantly, we observed the same enrichment of stress-response  
170 pathways and MAPK signaling in a fourth enzalutamide-resistant cell line, MDA-PCa-2b  
171 (**Fig. 3D, E**). MDA-PCa-2b is an independent androgen responsive and AR positive  
172 prostate cancer cell line. These data suggest that AR-positive enzaR cells exhibit unique  
173 genetic and transcriptional landscapes at the gene level that converge on p38 signaling  
174 and stress response signaling at the pathway level. These pathways are activated as a  
175 consequence of adaptation to enzalutamide and not an acute response, as a short-term (5  
176 day) treatment with enzalutamide does not activate this same p38-mediated response  
177 pathway (**Supplemental Fig. 6B**). This pathway-level convergence is reminiscent of  
178 convergent evolution observed in ecological contexts during resource depletion,  
179 including the conserved activation of the p38 MAPK pathway itself (Gatenby et al.,  
180 2011; Harrison et al., 2004; Li et al., 2011a).

181         Considering the convergent evolutionary behavior of the enzaR cells at the gene  
182 expression/signaling levels, we next attempted to better understand if these changes also  
183 promoted phenotypic convergence during the acquisition of enzalutamide resistance. To



184 do this, we first explored the phenotypic consequences of p38/MAPK pathway activation.  
185 The p38/MAPK pathway is a key regulator of the stress response (Igea and Nebreda,  
186 2015), and a regulator of tumor cell dormancy in many cancer types, including prostate  
187 cancer (Decker et al., 2017; Yu-Lee et al., 2018). Consistent with the role of p38 in a  
188 dormancy phenotype, enzaR cells exhibited a significant downregulation in the ratios of  
189 pERK:p38 $\alpha$ , an important indicator of the shift from a proliferative (high ERK) to  
190 dormant (high p38 $\alpha$ ) phenotype (**Supplemental Fig S7A**). Likewise, enzaR cells also  
191 upregulated p21, a cyclin dependent kinase inhibitor induced during stress response to  
192 regulate cell cycle progression (Sosa et al., 2011) (**Supplemental Fig S7B**). Interestingly,  
193 unlike enzalutamide-sensitive cells that induce beta-galactosidase activity, a marker of  
194 dormant cells, enzaR cells do not increase beta-galactosidase activity in response to  
195 enzalutamide treatment. However, enzaR cells have a higher baseline level of beta-  
196 galactosidase activity compared to enzalutamide sensitive cells (**Supplemental Fig**  
197 **S7C**). These data suggest that cells adapt to survival during enzalutamide treatment by  
198 regulating cell cycle progression and escaping from treatment induced dormancy  
199 programs.

200

## 201 **Activation of p38/MAPK is enriched in metastatic disease and drives cell growth in** 202 **prostate cancer**

203 Our results indicate that enzaR cells converge on p38 activation, which is  
204 functionally associated with dormancy and a pro-survival phenotype. Prior work has  
205 suggested activation of the p38 pathway in disseminated prostate and breast cancer cells  
206 from patients (Chery et al., 2014; Werden et al., 2016). Based on our preclinical data and

207 these observations, we hypothesized that p38 activation would be associated with  
208 aggressive and metastatic disease. To test our hypothesis we compared, by  
209 immunohistochemistry, 30 primary tumors and 20 metastatic biopsies from prostate  
210 cancer patients (Ware et al., 2016) for phospho-p38 expression. These tumors were all  
211 positive for AR expression. Importantly, as compared to localized prostate cancer,  
212 metastases show a strong activation of p38 (**Fig. 3F**).

213 To validate these findings, we performed kinase substrate enrichment analysis  
214 (KSEA) on our published phosphoproteomic dataset (Drake et al., 2016) consisting of 16  
215 metastatic prostate cancer patients. KSEA demonstrated significantly enriched  
216 hyperphosphorylation of p38 substrates in metastatic castration-resistant prostate cancer  
217 (CRPC) tissues compared to localized hormone-naïve prostate cancer tissue (**Fig. 3G**,  
218 **Supplemental Table 1**). Independent data sets analysis shows p38 activation as the  
219 central hub of cell signaling convergence in human prostate cancer metastases,  
220 particularly during the evolution of metastasis and hormone therapy resistance.

221 Next, we tested whether inhibition of p38 signaling impacted cell growth in our  
222 models of enzalutamide resistance. In all enzaR cell line models, treatment with a small  
223 molecule p38 inhibitor (SB203580) led to a significant decrease in cell proliferation over  
224 time (**Fig. 4A-C**). These data highlight the p38 signaling pathway as important and  
225 activated in enzalutamide resistant prostate cancer.

226

227

228

229 **Activation of p38/MAPK promotes sustained AR signaling in the presence of**  
230 **enzalutamide**

231 Previous studies have shown that enzalutamide induces lineage reprogramming  
232 toward an AR null, neuroendocrine-like phenotype (Ku et al., 2017; Mu et al., 2017;  
233 Paranjape et al., 2016). However, neither the RNA-Seq nor qRT-PCR analyses revealed  
234 consistent changes in neuroendocrine biomarkers across our four paired enzalutamide  
235 resistant cell lines (**Supplemental Fig. 4**). On the contrary, all enzaR cell line models  
236 maintain AR protein expression (**Fig 2C, D**), indicating that phenotypic shifts to AR-null  
237 (Bluemn et al., 2017) or neuroendocrine lineages (Aparicio et al., 2011; Dardenne et al.,  
238 2016) have not occurred in our four model systems. Thus, our models may recapitulate  
239 the common occurrence of AR positive metastatic castration-resistant prostate cancer  
240 post-abiraterone/enzalutamide, observed in the majority (>60-70%) of men with lethal  
241 prostate cancer (Bluemn et al., 2017) (**Fig. 2**). Consistent with this, treatment with  
242 androgen (R1881) induces AR activity as measured by PSA expression (**Fig. 4D-F**),  
243 implying that enzaR cells remain androgen dependent and responsive to AR signaling.

244 To understand how enzalutamide-induced p38 activation may impact AR activity,  
245 we treated control and enzaR cells with the p38 inhibitor, SB203580. Inhibition of p38  
246 led to dramatic down regulation of AR activity in both sensitive and resistant cell lines,  
247 which suggests p38 plays a role in promoting AR activity in the setting of CRPC.  
248 Additionally, treatment with the p38 inhibitor, SB203580, partially blocked androgen-  
249 stimulated *PSA* and *NKX3.1* expression (**Fig. 4D-F, S6A**). Taken together, our data  
250 indicates p38 activation promotes convergent AR-dependent enzalutamide resistance, at  
251 least in part, by facilitating persistent AR activation.

252 To further validate the importance of p38 signaling in promoting enzalutamide  
253 resistant growth we performed population-level p38 knockout or p38 constitutive  
254 activation in LNCaP and LN95 cells. CRISPR/Cas9-mediated knockout of p38 delayed  
255 outgrowth of enzalutamide-resistant cells (**Fig. 5A, B**) and constitutive activation of p38  
256 promoted resistance to enzalutamide treatment (**Fig. 5C, D**). Likewise, treatment with the  
257 p38 inhibitor, SB203560, *in vivo* significantly reduced the growth of enzaR xenografts  
258 (**Fig. 4G**). Consistent with the decreased tumor growth over time, histology from tumor  
259 tissues treated with the p38 inhibitor indicated acute inflammation and necrosis with little  
260 viable tumor compared to diluent (control) treated tumors (**Fig 4H**). Together, these data  
261 suggest a causal mechanistic relationship between p38 activation and acquired resistance  
262 to AR inhibition in prostate cancer that can be overcome by p38 blockade *in vivo* and  
263

#### 264 **A p38/MAPK axis induces pro-metastatic and immuno-evasive phenotypes**

265 The observation that p38 activity was increased in metastatic tissues suggested  
266 that p38 may facilitate metastatic progression or is upregulated during the metastatic  
267 cascade. To better understand the mechanisms by which p38 activity may promote  
268 metastasis, we interrogated known drivers of metastatic prostate cancer. One of these  
269 drivers of metastasis is Snail, a master regulator of epithelial plasticity that promotes  
270 migration and invasion and is strongly expressed in 100% of metastatic prostate cancer  
271 biopsies (Ware et al., 2016). We have previously shown that Snail activates AR nuclear  
272 localization to drive plasticity, invasion, and enzalutamide resistance (Ware et al., 2016).  
273 Snail has also been implicated in epithelial plasticity and loss of AR activity in prostate  
274 cancer models of AR therapy resistance as well through direct binding to the AR gene

275 locus (Miao et al., 2017). We observed Snail upregulation in the p38-activated,  
276 enzalutamide-resistant cell lines (**Fig. 6A**) as compared to enzalutamide sensitive models,  
277 suggesting that enzalutamide-induced p38 activation induces Snail upregulation.  
278 Consistent with Snail upregulation, enzalutamide-resistant cells displayed an increase in  
279 repressive phosphorylation at serine 9 on the upstream Snail destabilizing protein and  
280 known p38 target, GSK3 $\beta$  (Bikkavilli et al., 2008) (**Supplemental Fig. S8A**). These  
281 results provide a mechanistic link connecting p38 activation to increased Snail through  
282 suppression of GSK3 $\beta$  activity, which may lead directly to altered AR activity and  
283 nuclear localization.

284         Prior studies have demonstrated an association between enzalutamide resistance  
285 and PD-L1 expression on tumor and immune cells, as well as the potential benefits of  
286 combined enzalutamide and PD-1 inhibition (Bishop et al., 2015; Graff et al., 2016;  
287 Zhang et al., 2018), suggesting a connection between AR inhibition and immune evasion.  
288 Interestingly, we also observed a significant positive correlation in gene expression data  
289 in prostate cancer tissues from The Cancer Genome Atlas between Snail expression and  
290 expression of the immune checkpoint molecule, PD-L1 (**Fig. 6B**). To identify Snail as an  
291 effector molecule upstream of PD-L1 expression, we used an inducible system to activate  
292 Snail in LN95 prostate cancer cells (Ware et al., 2016). Snail activation led to  
293 upregulation of PD-L1 mRNA and protein (**Fig. 6C, D**). We also noted common  
294 upregulation of PD-L1 by reverse phase protein array analysis, qPCR, and western  
295 blotting in all enzaR models as compared to the parental models (**Fig. 7A-C**).

296         Given the positive relationship between p38 phosphorylation, PD-L1, and Snail  
297 expression in both our models and clinical samples, we next sought to determine the

298 mechanistic links between p38, Snail, and PD-L1. To do this we assessed the  
299 consequence of inhibiting or activating p38 signaling on Snail and PD-L1 expression.  
300 Remarkably, p38 $\alpha$  knockdown led to downregulation of both Snail and PD-L1 (**Fig. 7D**)  
301 and constitutive activation of p38 $\alpha$  induced Snail and PD-L1 upregulation (**Fig. 7E**).  
302 These experiments suggest that p38 activation can promote a pro-metastatic, immune  
303 evasive phenotype by stabilizing Snail expression (Ryu et al., 2019) and leading to  
304 downstream PD-L1 upregulation. Together, our data suggest that p38 may provide a  
305 common mechanistic explanation for evolutionary convergence of cancer cell survival,  
306 metastasis, and immune evasion phenotypes in AR positive metastatic castration-resistant  
307 prostate cancer, which could be therapeutically targeted through p38 blockade to treat  
308 men with hormone therapy resistant prostate cancer (**Fig. 7F**).

309

## 310 **Discussion**

### 311 **Hormone therapy-resistant prostate cancer cells undergo convergent evolution onto** 312 **a pro-survival, metastatic, immuno-evasive gene regulatory network**

313 Our integrated, multi-omics analysis of the evolution of enzalutamide resistance in  
314 prostate cancer revealed consistent activation of the p38/Snail/PD-L1 gene regulatory  
315 network in the context of persistent AR expression and activity. Activation of this gene  
316 expression pathway was observed in the face of heterogeneous and unique acquired  
317 genetic landscapes across the diverse model systems tested. Likewise, our current and  
318 previous clinical analyses revealed upregulation of both p38 and Snail in metastases from  
319 metastatic castration-resistant prostate patients ([Ware et al., 2016](#)), all of which are likely  
320 to harbor substantial spatial and temporal genetic heterogeneity. This phenotypic

321 similarity is reminiscent of convergent evolution in natural systems in which strong  
322 selective pressures lead to optimal adaptive phenotypes, such as the independent  
323 evolution of flight and the loss of sight in low-light environments across multiple,  
324 genetically-distinct taxonomic lineages (Gatenby et al., 2011). These results suggest that,  
325 in addition to known genetic drivers of resistance (Azad et al., 2015; Ku et al., 2017;  
326 Mazrooei et al., 2019; Mu et al., 2017; Romanel et al., 2015), the evolution of resistance  
327 can also be mediated by gene regulatory phenotypes rather than any common genetic  
328 driver. This convergent evolution into critical molecular driver pathways may be  
329 overlooked through the analysis of DNA or RNA-based sequencing approaches in  
330 isolation.

331

### 332 **The p38/Snail signaling axis promotes cell-intrinsic enzalutamide resistance and** 333 **metastasis**

334 Eukaryotic cells have evolved to respond to environmental stressors through a robust  
335 and highly-coordinated stress response system (Sun and Zhou, 2018). This system is  
336 dynamically regulated through multi-level regulation of gene expression networks, which  
337 induce reversible phenotypic changes to enable cellular responses.

338 In the context of cancer, cells encounter a range of environmental stressors, such as  
339 hypoxia, reactive oxygen species, glucose and nutrient deprivation, and mechanical stress  
340 (reviewed in (Chen and Xie, 2018)). In addition to these microenvironmental factors,  
341 cancer therapy dramatically alters the environment and induces a range of cellular  
342 responses to enable cell survival in the face of therapy-induced resource depletion. One  
343 of these key cellular responses is p38/MAPK pathway activation. The p38 pathway is an

344 evolutionarily-conserved stress response system (Li et al., 2011a), which is responsible  
345 for transducing stress signals, such as DNA damage, reactive oxygen species, cytokines,  
346 and changes in osmotic pressure, from the extracellular environment to activate  
347 transcriptional response programs (Coulthard et al., 2009). Activation of the p38/MAPK  
348 pathway has been implicated in numerous cancers, including metastatic prostate cancer  
349 (Drake et al., 2016; Khandrika et al., 2009; Koul et al., 2013). Consistent with its role in  
350 mediating cellular stress responses, we found that p38 promotes cell-intrinsic resistance  
351 to enzalutamide by promoting reactivation of AR activity. Mechanistically, we observed  
352 increased phosphorylation of GSK3 $\beta$  at serine 9 (**Supplemental Fig. 8**), which leads to  
353 inactivation of GSK3 $\beta$  activity and induces upregulation of Snail. This model is  
354 supported by previous reports, which have demonstrated that p38 inhibits GSK3 $\beta$   
355 through phosphorylation (Thornton et al., 2008), which can lead to Snail stabilization  
356 (Zhou et al., 2004). Importantly, we have demonstrated that Snail contributes to  
357 enzalutamide resistance via upregulation of AR activity (Ware et al., 2016).

358 In addition to its functions in enzalutamide resistance, p38 and Snail also drive a pro-  
359 metastatic and death-resistant phenotype in prostate cancer. Consistent with this,  
360 inhibition of p38 leads to reduced survival, clonogenicity, and invasion in prostate cancer  
361 cells (Khandrika et al., 2009). Others have also shown that activation of p38 induces  
362 TGF- $\beta$ -mediated Snail upregulation and invasion (Medici et al., 2011). Similarly, we  
363 previously demonstrated that Snail is strongly elevated in metastatic, castration-resistant  
364 prostate cancer compared to primary prostate cancer (Ware et al., 2016). We also found  
365 that Snail activation leads to enhanced migration and invasion of prostate cancer cells



366 (Ware et al., 2016). In the current study, we define a mechanistic connection between the  
367 p38 pathway and Snail that drives enzalutamide resistance and metastasis.

368

### 369 **The p38/Snail axis promotes cell-extrinsic immune evasion**

370 Coupled with their roles in enzalutamide resistance, our data suggest the  
371 p38/Snail pathway may also contribute to immune evasion by upregulating the immune  
372 checkpoint molecule, PD-L1. In addition, enzalutamide exposure alone leads to  
373 upregulation of Snail and PD-L1 (Bishop et al., 2015), and our data demonstrates Snail  
374 activation can upregulate PD-L1 expression. Consistent with this, PD-L1 is upregulated  
375 on melanoma cells through activation of p38 (Noh et al., 2015), providing further strong  
376 support for our hypothesis that the p38/Snail axis drives PD-L1 expression. Similarly, in  
377 both chronic viral infections and cancer, p38 activation has been linked to inhibition of  
378 Stimulator of IFN genes (STING) expression, enhanced CXCR2-mediated myeloid  
379 derived suppressor cell activity, and immune evasion (Chen et al., 2017; Wang et al.,  
380 2006; Zhang et al., 2017). Together, these findings suggest that p38 and/or immune  
381 checkpoint blockade may be therapeutically efficacious in the post-enzalutamide setting.  
382 In support of this notion, Graff et al. observed rapid reductions in prostate specific  
383 antigen and radiographic responses in approximately ~20% of men with enzalutamide  
384 resistant metastatic castration-resistant prostate treated with a combination of the anti-  
385 PD-1 therapy pembrolizumab and enzalutamide (Graff et al., 2016; Graff et al., 2018).  
386 Furthermore, PD-L1 can indirectly activate p38 through DNA-PKcs, which promotes  
387 chemoresistance (Wu et al., 2018), and therefore suggests a common feed forward loop  
388 that may connect PD-L1 expression, DNA repair and pro-metastatic pathways (Goodwin

389 et al., 2015), and convergent p38 activity. Interestingly, PD-L1 expression in the tumor  
390 and tumor microenvironment is also associated with poor outcomes and an aggressive  
391 and metastatic phenotype in many cancer subtypes (Kim et al., 2016; Wang et al., 2018).

392

### 393 **Beyond enzalutamide resistance**

394 The current work highlights the role of the p38 pathway in promoting  
395 enzalutamide resistance in prostate cancer. However, the importance of the p38 pathway  
396 as a general stress response to any unfavorable environment may suggest the potential of  
397 targeting the p38 pathway in other resource-limiting settings in cancer. Indeed, our  
398 clinical data suggest that the p38/Snail pathway is induced during metastasis even prior to  
399 androgen deprivation or enzalutamide treatment. Therefore, tumor cells undergoing a  
400 stress response independent of enzalutamide or prostate cancer may also rely on p38  
401 activation to persist and survive in ecologically-unfavorable environments, such as the  
402 hostile environment of the bloodstream during dissemination, or during treatments that  
403 target other oncogenic drivers across different cancer types. For example, p38 activity has  
404 been shown in both breast and lung cancer to promote chemotherapy resistance (Flem-  
405 Karlsen et al., 2019; Liu et al., 2016; Lu et al., 2018). Activation of p38 has also been  
406 linked to DNA repair (Canovas et al., 2018), which is also enriched in our models of  
407 enzalutamide resistance (**Fig. 3B**). Therefore, p38 inhibition in combination with  
408 chemotherapeutic drugs that induce chromosome instability may have therapeutic  
409 potential. These data suggest there may be clinical utility in re-purposing p38 inhibitors  
410 for the investigation of reversing treatment resistance in metastatic castration-resistant  
411 prostate and other cancers (**Fig. 7F**).

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423 thank Medivation/Astellas for providing the AR inhibitor, enzalutamide.

424

425 **Author Contributions**

426 Conceptualization, KEW, JAS, AJA; Methodology, KEW, JAS, AJA, DLC, MP, EFP,  
427 ZS, JMD, LAC, WCF; Investigation, KEW, JAS, SG, JE, GK, BJP, RGA, DR, BCT,  
428 AA, MP; Writing-Original Draft, KEW, JAS, AJA; Visualization, MUS, JAS, KEW;  
429 Resources, JAS, AJA, KEW, JF, SRP, TZ, SG, JMD

430

431 **Declaration of Interests**

432 AJA receives consulting income and research support to Duke from Pfizer/Astellas,  
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439 consultant to and shareholder of Avant Diagnostics, Inc and Perthera, Inc. All other  
440 authors declare no competing interests.

441

## 442 **Figure Titles and Legends**

### 443 **Figure 1**

444 **Model of ecological responses to loss of AR signaling.** Adaptation to enzalutamide  
445 centers around three phenotypes: 1) Pro-survival stress response; 2) Immune evasion; and  
446 3) Metastasis.

447

### 448 **Figure 2**

449 **Enzalutamide resistant cell lines retain AR expression and do not undergo a**  
450 **NEPC/EMT/Stem transition. A.** Development of enzalutamide resistant cell lines. **B.**  
451 Control (ctrl) and enzaR cell lines were treated for 10 days with vehicle (DMSO) or two  
452 doses of enzalutamide (25 $\mu$ M or 50 $\mu$ M), and cell growth and apoptosis were measured.  
453 \*= p<0.05; student's t-test. Error bars represent the standard error of the mean. **C-D.** AR-  
454 FL and AR-V7 mRNA (**C**) and protein expression (**D**) measured by qRT-PCR and  
455 western blot, respectively, \*p<0.05; student's t-test. Mean cell values are plotted along  
456 with standard error of the mean. **E.** Summary table characterizing the diversity of known

457 resistance mechanisms in enzalutamide resistant cell lines. ADT, androgen deprivation  
458 therapy; Enza, enzalutamide; EnzaR, enzalutamide resistant.

459

460 **Figure 3**

461 **Diverse gene expression during enzalutamide resistance converges on activation of**  
462 **stress response MAPK p38. A.** Venn diagrams representing common gene expression  
463 alterations by RNA-Seq or protein activation by proteomics analysis (top). Enriched  
464 pathways in each matched enzaR cell line using gene set enrichment analysis (bottom)  
465 from RNA-Seq data. **B.** Combined RNA-Seq analysis from all enzalutamide-resistant  
466 lines identified p38/MAPK signaling and stress-related pathways. **C.** Volcano plot  
467 representing changes in phosphorylation from reverse phase protein array analysis. **D.**  
468 RNA-Seq analysis for pathway enrichment scores in MDA-PCa-2b cells. **E.** Western blot  
469 analysis of phosph-p38 in MDA-PCa-2b cells. **F.** Immunohistochemical staining of  
470 phospho-p38 is strongly enriched in metastatic tissues compared to localized samples.  
471 Representative images show phospho-p38 staining in localized and metastatic prostate  
472 cancer tissues. **G.** Kinase substrate enrichment analysis (KSEA) of p-p38 revealed an  
473 enrichment of p38 substrates in metastatic CRPC patients using published  
474 phosphoproteomic datasets.

475

476 **Figure 4**

477 **p38 inhibition is important for cell growth in EnzaR cell lines. A-C.** Control or enzaR  
478 cells were cultured in media containing DMSO or 25 $\mu$ M SB203580, and cell confluence  
479 was quantified using the IncuCyte basic analyzer. **D-F.** Control or enzaR cells, treated

480 with androgen (R1881), p38 inhibitor (SB203580), or the combination of R1881 and  
481 SB203580 were analyzed for PSA mRNA expression. Amounts were determined by  
482 qRT-PCR and normalized to GAPDH, \* =  $p < 0.05$ ; student's t-test.

483

#### 484 **Figure 5**

485 **Molecular alteration of p38 expression regulates enzalutamide sensitivity.** **A.** EnzaR  
486 cells with p38 knockout were cultured and cell confluence was quantified using the  
487 IncuCyte basic analyzer. Knockout of p38 in enzaR cell line populations decreases cell  
488 proliferation of LNCaP (A) and LN95 cells (B). **C.D.** Enzalutamide sensitive cells  
489 engineered to overexpress p38 were cultured and cell confluence was quantified as  
490 above. Overexpression of p38 $\alpha$  in enzalutamide sensitive control cell lines increases cell  
491 growth in response to enzalutamide treatment. **E.** Mice were injected subcutaneously  
492 with  $8 \times 10^6$  CS2-enzaR cells and treated with diluent or 15 mg/kg of p38 inhibitor  
493 (SB203580). Tumor growth was measured weekly over 13 weeks. \* =  $p < 0.05$ ; student's  
494 t-test. **F.** Representative 10X images of H&E staining from xenograft tumors collected  
495 after treatment with diluent or p38 inhibitor (15 mg/kg SB203580) demonstrating viable  
496 (top) and necrotic (bottom) tumor.

497

#### 498 **Figure 6**

499 **PD-L1 expression correlates with Snail expression and activity during enzalutamide**  
500 **resistance.** **A.** Western blot analysis of phospho-p38 and Snail expression in control and  
501 enzaR cell lines. GAPDH is used as a loading control. **B.** Analysis of RNA-Seq data from  
502 The Cancer Genome Atlas reveals a significant positive correlation between Snail and

503 PD-L1 expression in prostate cancer tissues. **C.** PD-L1 mRNA expression in LN95 cells  
504 with and without Snail activation. **D.** Snail and PD-L1 protein expression in LN95 cells  
505 with and without Snail activation. Blue: Hoechst stained nuclei; Red stained Snail; Green  
506 stained PD-L1.

507

## 508 **Figure 7**

509 **p38 is a druggable target upstream of Snail and PD-L1.** **A.** PD-L1 protein expression  
510 is significantly increased in enzaR cells by reverse phase protein array analysis. **B-C.** PD-  
511 L1 mRNA (**B**) and protein (**C**) expression measured by qPCR and western blot,  
512 respectively, in enzaR cells. **D.** Western blot analysis of p38, Snail, and PD-L1 in LN95  
513 enzaR cells with p38 $\alpha$  knockout. NaKATPase is used as a loading control. **E.** Western  
514 blot analysis of p38, Snail, and PD-L1 in LN95 control cells with p38 $\alpha$  activation.  
515 NaKATPase is used as a loading control. **F.** Model of p38 as a druggable target upstream  
516 of Snail and PD-L1 induced during resistance to enzalutamide.

517

## 518 **Materials and Methods**

### 519 **Patient samples**

520 For metastatic prostate cancer samples (n=20) and a second cohort of localized  
521 prostate cancer samples (n=30), formalin fixed metastatic tissue was collected from the  
522 Duke University pathology department and Duke Cancer Institute Biorepository Core  
523 under a separate Duke IRB approved protocol. Clinical data on prior therapy and  
524 metastatic site were collected.

525

526 **Cell lines**

527 MDA-PCa-2b and LNCaP cells were obtained from ATCC using the Duke University  
528 Cell Culture Facility. MDA-PCa-2b cells were cultured in F12-K media supplemented  
529 with 20% fetal bovine serum (Sigma), 1% penicillin/streptomycin streptomycin (Life  
530 Technologies), cholera toxin, epidermal growth factor, phosphoethanolamine,  
531 hydrocortisone, sodium selenite, and insulin as recommended by ATCC. LNCaP cells  
532 were cultured in RPMI containing 10% fetal bovine serum and 1%  
533 penicillin/streptomycin. LNCaP95 cells were kindly provided by Dr. Scott Dehm  
534 (University of Minnesota) and are reported in previous studies (Liu et al., 2013; Ware et  
535 al., 2016). LNCaP95 and CS2 cells are androgen-independent cell lines derived from the  
536 parental LNCaP cells. LNCaP95 and CS2 were cultured in RPMI containing 10%  
537 charcoal stripped fetal bovine serum and 1% penicillin/streptomycin. Resistant cell  
538 populations were cultured with the addition of 50  $\mu$ M enzalutamide (provided by  
539 Medivation/Astellas). Enzalutamide resistant cells (EnzaR cells) were generated by  
540 chronic culture with increasing doses of enzalutamide to a concentration of 50  $\mu$ M. Cells  
541 were authenticated and re-authenticated following enzalutamide resistant through  
542 sequencing including the presence of known LNCaP AR ligand binding domain mutation  
543 T877A. Cells stably expressing inducible Snail (Addgene plasmid #18798), constitutively  
544 active p38 or MAPK14 gRNA targets were generated by transduction of cells as  
545 described previously (Mani et al., 2008).

546

547

548



549 **Cell growth and viability assays**

550 Control and Enza-R cells were cultured in media containing DMSO (Sigma) or 50  $\mu$ M  
551 enzalutamide for at least one week. Cells were counted using the Countess II (Life  
552 Technologies), 2500 cells were seeded in a 96-well plate. Cell confluence was monitored  
553 using the IncuCyte live cell analysis system (Essen Biosciences) and standard error of the  
554 mean (SEM) was calculated from triplicate wells. For cells stably expressing p38 or  
555 MAPK14 gRNA, cells were treated with DMSO or 25  $\mu$ M of SB203580 (selleckchem)  
556 and monitored as above. For cell viability, apoptosis was measured after 10 days of drug  
557 treatment using the IncuCyte Caspase-3/7 green apoptosis assay reagent and quantified  
558 using the IncuCyte basic analyzer.

559

560 **RNA-seq**

561 Total RNA was isolated using the Quick-RNA Miniprep kit (Zymo Research). RNA-seq,  
562 total RNA was submitted to the Duke Center for Genomic and Computational Biology  
563 core for sample preparation, sequencing, and analysis. RNA-seq data was processed  
564 using the TrimGalore toolkit  
565 ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore)) which employs  
566 Cutadapt<sup>1</sup> to trim low quality bases and Illumina sequencing adapters from the 3' end of  
567 the reads. Only pairs where both reads were 20nt or longer were kept for further analysis.  
568 Reads were mapped to the GRCh37v73 version of the human genome and transcriptome<sup>2</sup>  
569 using the STAR RNA-seq alignment tool<sup>3</sup>. Reads were kept for subsequent analysis if  
570 they mapped to a single genomic location. Gene counts were compiled using the HTSeq  
571 tool (<http://www-huber.embl.de/users/anders/HTSeq/>). Only genes that had at least 40

572 reads in any given library were used in subsequent analysis. Normalization and  
573 differential expression was carried out using the EdgeR<sup>4</sup> Bioconductor<sup>5</sup> package with the  
574 R statistical programming environment ([www.r-project.org](http://www.r-project.org)). A negative binomial  
575 generalized log-linear model<sup>6</sup> was used to identify differentially expressed genes between  
576 the different genotypes when comparing against specific control samples. Enriched  
577 pathways were determined by GSEA<sup>7</sup> for each comparison.

578

### 579 **Real-Time quantitative RT-PCR**

580 For qPCR, total RNA was reverse transcribed using the High-Capacity cDNA Reverse  
581 Transcription Kit (Life Technologies). Aliquots of 5-fold diluted reverse transcription  
582 reactions were subjected to quantitative (q)PCR with KAPA SYBR FAST master mix  
583 using the Vii7 real time-PCR detection system (Applied Biosystems). GAPDH mRNA  
584 levels were measured for normalization, and the data are presented as “Relative  
585 Expression”. A complete list of primer sequences is provided in the supplementary text.

586

### 587 **TCGA Analysis**

588 The results published here are in part based upon data generated by The Cancer Genome  
589 Atlas (TCGA) Research Network: <http://cancergenome.nih.gov>. Data available from  
590 TCGA was analyzed using the Kruskal Wallis test to evaluate the correlation between  
591 Snail expression and PD-L1 expression from prostate cancer patients.

592

593

594

595 **Reverse phase protein array**

596 Cells were seeded (300,000/well) in 6-well plates and allowed to incubate for 5 days.

597 Cells were then rinsed with PBS, flash frozen, and analyzed as previously described

598 (Baldelli et al., 2017; Pierobon et al., 2017).

599

600 **Immunoblot analyses**

601 For immunoblot analysis cells extracts were mixed with SDS sample buffer and

602 submitted to SDS-PAGE. Following electrophoretic transfer onto nitrocellulose, the

603 filters were blocked in Starting Block (Thermo), incubated with antibodies and developed

604 using the Odyssey-FC imager (LI-COR).

605 A complete list of primary antibodies and their dilutions is provided in the

606 supplementary text.

607

608 **Immunofluorescence staining**

609 For cells expressing inducible Snail, cells were pretreated with ethanol (EtOH) or 4OHT.

610 For immunofluorescence (IF) staining, cells were fixed in 4% PFA, permeabilized with

611 0.2% Triton X-100, and stained with Hoechst. Cells were blocked with 5% bovine serum

612 albumin (BSA, Sigma) prior to incubation with primary antibodies. Cells were incubated

613 in Alexa Fluor secondary antibodies (Life Technologies) and then imaged on an inverted

614 Olympus IX 73 epifluorescence microscope.

615

616

617

## 618 **Immunohistochemistry**

619 We performed antibody optimization and analytic validation for all antibodies as  
620 previously described (Armstrong et al., 2016). An expert prostate cancer pathologist  
621 blinded to outcomes evaluated antibody staining in parallel with hematoxylin and eosin.  
622 Scoring of each biomarker used a 0 to 3 scale for both intensity and a <25%, <50%,  
623 <75%, <100% scale for frequency of expression in each tumor sample.

624

## 625 **Animal Experiments**

626 Six to eight week male mice were injected subcutaneously with  $8 \times 10^6$  CS2-enzaR cells.  
627 Prior to injection cells were resuspended in 50% matrigel supplemented with  
628 10% FBS/RPMI. Mice were treated with diluent or 15 mg/kg of p38 inhibitor  
629 (SB203580). Tumor growth was measured using calipers weekly over 13 weeks.

630

## 631 **Statistical Analysis**

632 Data are shown as means  $\pm$  SEM. Student's t-test or multiple group comparison was  
633 performed by one-way ANOVA followed by the Sidak method for comparison of means.  
634  $P \leq 0.05$  is considered significant. Differences in phospho-p38 expression between  
635 localized and metastatic samples were analyzed using a Chi-square test. Data available  
636 through the TCGA was analyzed using the Kruskal Wallis test using JMP (version pro  
637 12). All other analyses were performed using Prism (version 8.0d).

638

639

640

641 **Supplemental Information Titles and Legends**

642 **Figure 1**

643 **Treatment with enzalutamide enriches for the F876L agonist mutation in LNCaP**

644 **enzaR cells.** Alignment of RNA-seq tracks using the integrative genomic viewer.

645

646 **Figure 2**

647 **Copy number alterations in control versus enzaR cell lines.** Array comparative

648 genomic hybridization from genomic DNA isolated from paired enzaR cell line models.

649 Alteration losses and gains greater than one log are highlighted in blue or red,

650 respectively.

651

652 **Figure 3**

653 **EnzaR cell lines do not undergo NEPC/Stemness transitions. A.** Representative

654 images of enzalutamide sensitive and resistant cell lines. **B.** qPCR expression analysis of

655 driver genes involved in neuroendocrine and stem cell pathways.

656

657 **Figure 4**

658 **Copy number alterations do not converge between enzaR cell lines.** Venn diagrams

659 comparing copy number gains and losses between control and enzaR cell lines.

660

661 **Figure 5**

662 **EnzaR cell lines acquire very few nucleotide variants during adaptation to**

663 **enzalutamide. A.** Venn diagrams comparing nucleotide variants between paired control

664 and enzaR cell lines. **B.** Venn diagram comparing common nucleotide variants observed  
665 in enzaR compared to control cell lines.

666

667 **Figure 6**

668 **AR activity is inhibited by p38 inhibition. A.** Control or enzaR cells, treated with  
669 androgen (R1881), p38 inhibitor (SB203580), or the combination of R1881 and  
670 SB203580 were analyzed for NKX3.1 mRNA expression. Amounts were determined by  
671 qRT-PCR and normalized to GAPDH. **B.** Western blot analysis of phospho-p38  
672 expression in control and enzaR cell lines with acute enzalutamide (10  $\mu$ M) treatment.

673

674 **Figure 7**

675 **EnzaR cells display markers of a dormant phenotype. A.** Ratios of phospho-ERK to  
676 phospho-p38 protein levels. **B.** Western blot analysis of p21 expression in control and  
677 enzaR cell line models. **C.** Beta-galactosidase activity in response to enzalutamide  
678 treatment in LNCaP cells.

679

680 **Figure 8**

681 **Molecular alteration of p38 expression regulates enzalutamide sensitivity. A.** GSK3 $\beta$   
682 serine 9 phosphorylation is significantly increased in enzaR cells by reverse phase protein  
683 array analysis.

684

685 **References**

686

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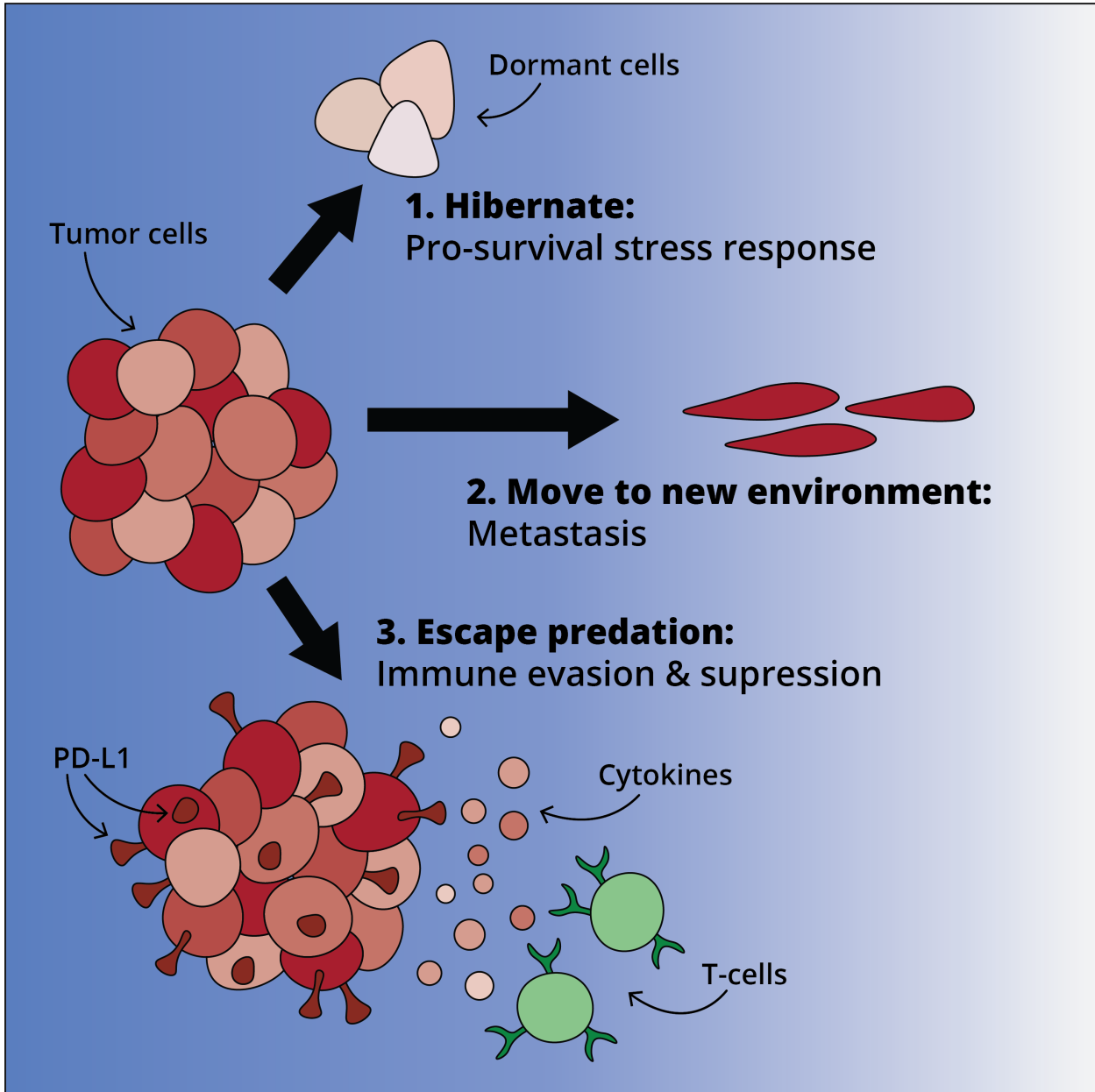
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Figure 1

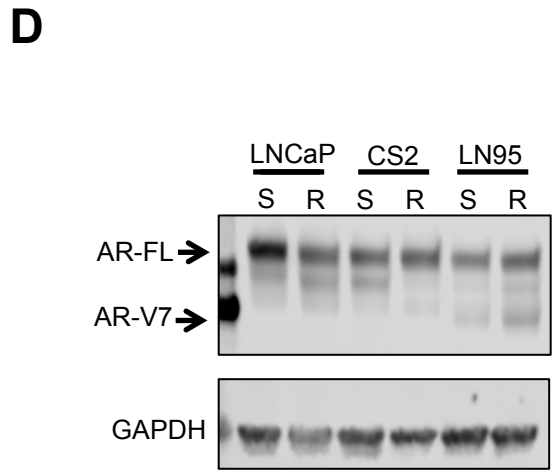
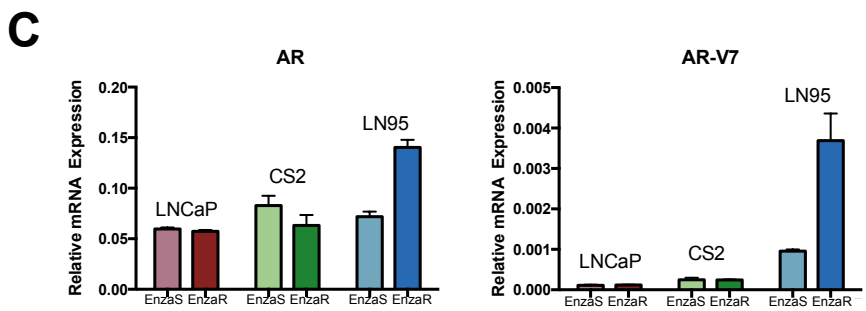
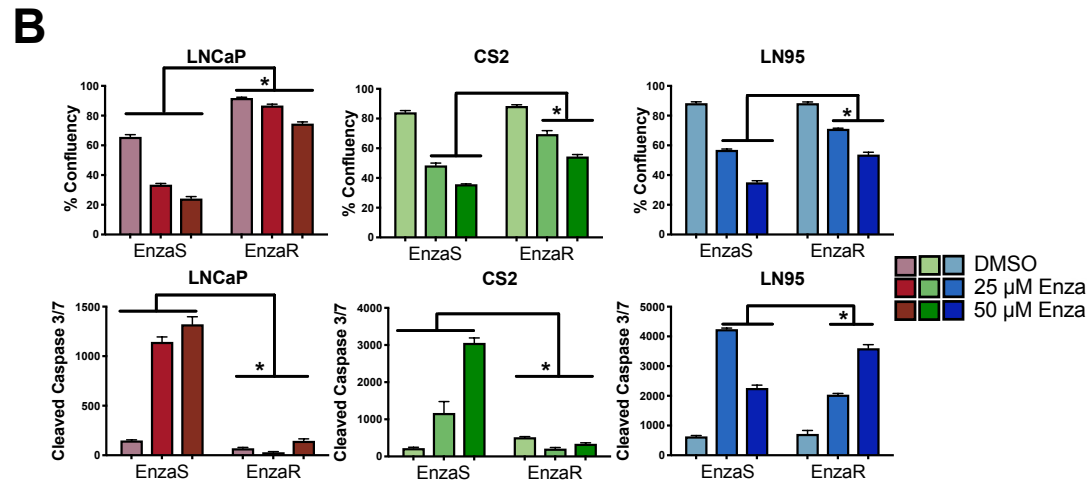
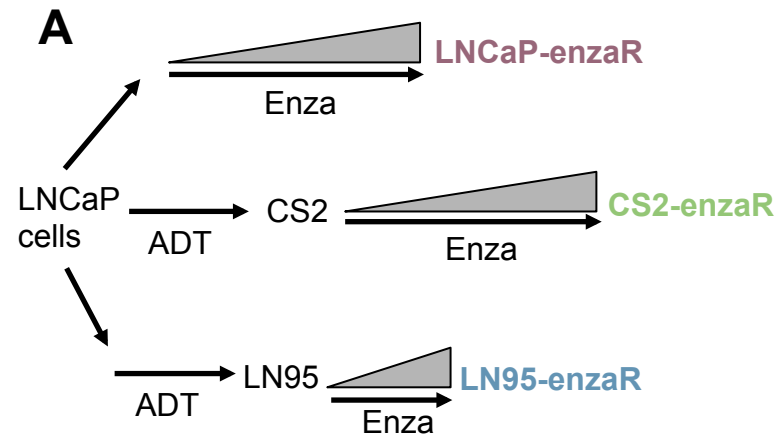


Unfavorable environment



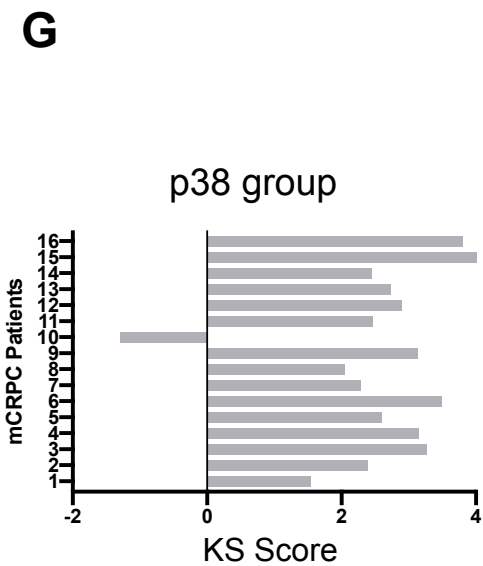
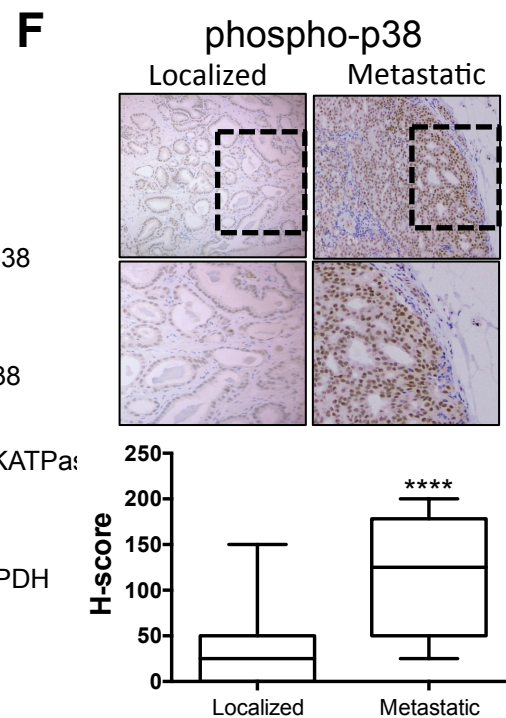
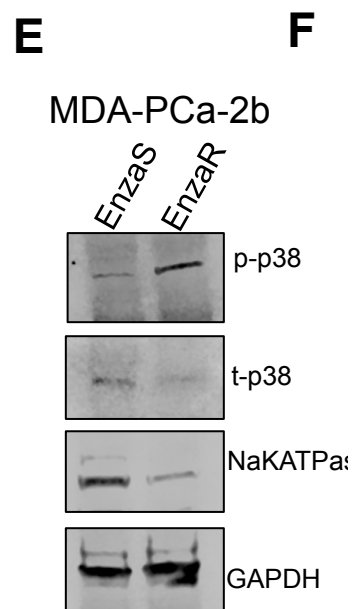
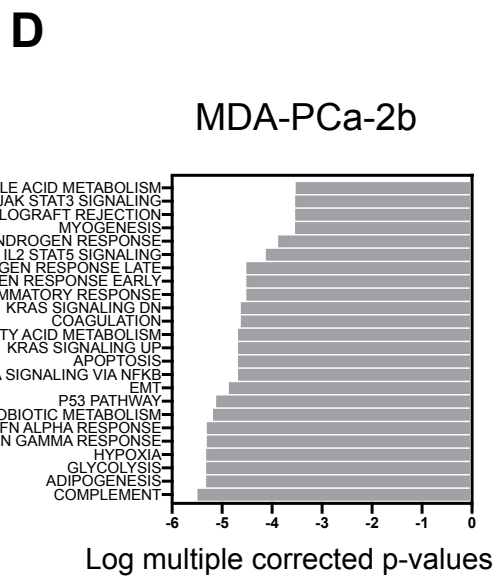
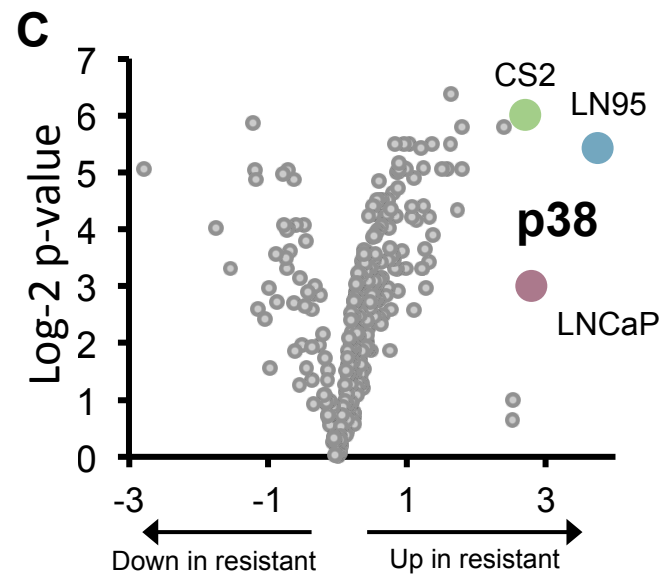
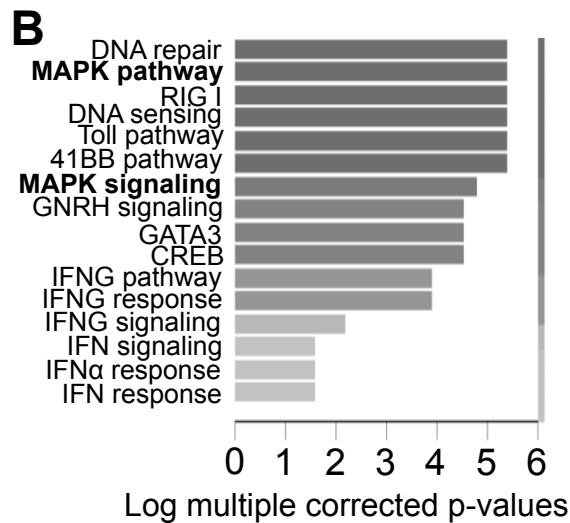
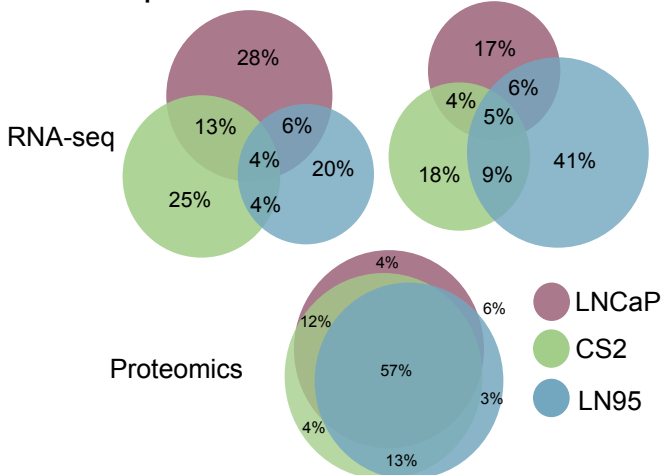
Favorable environment

**Figure 2**

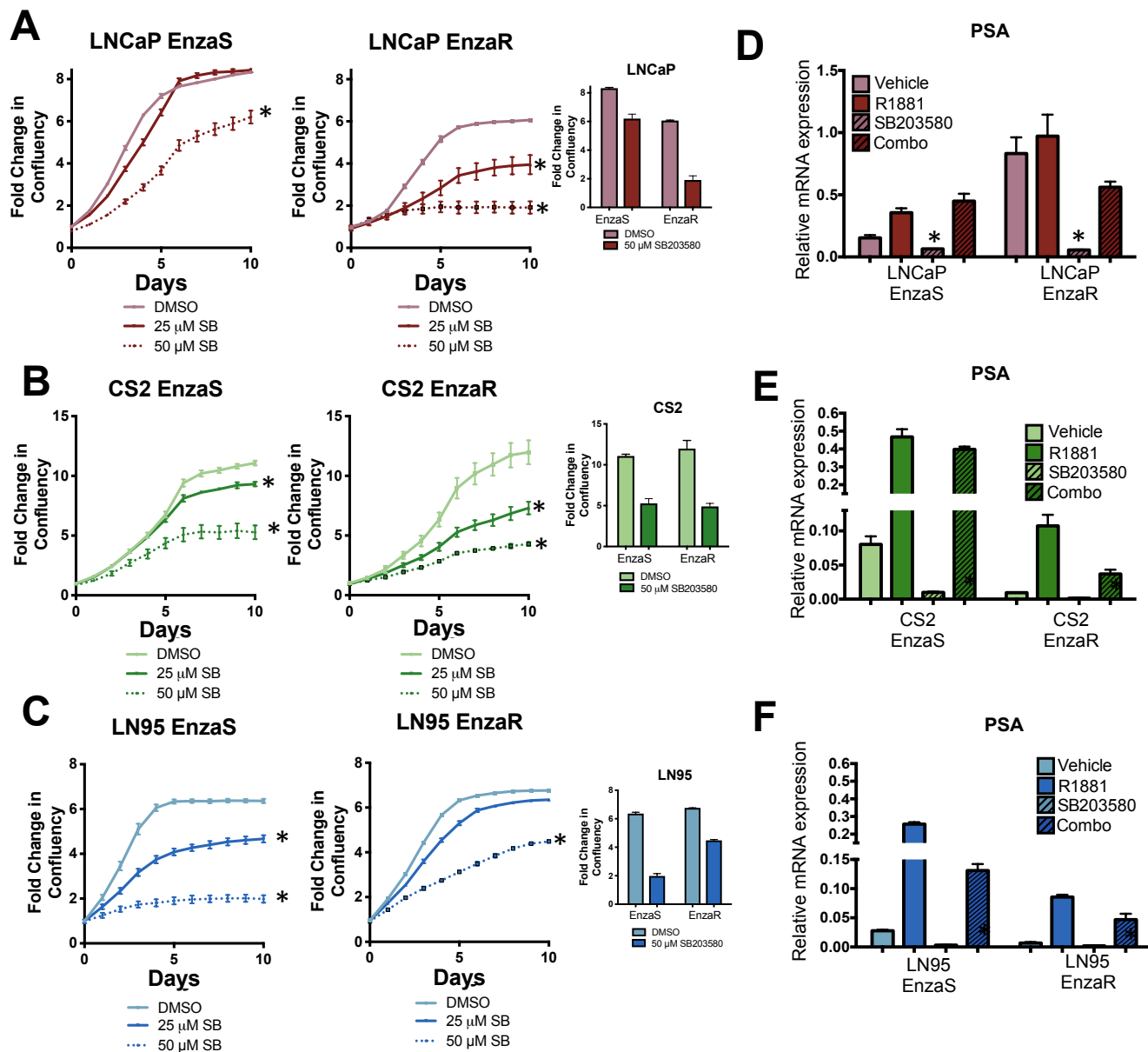


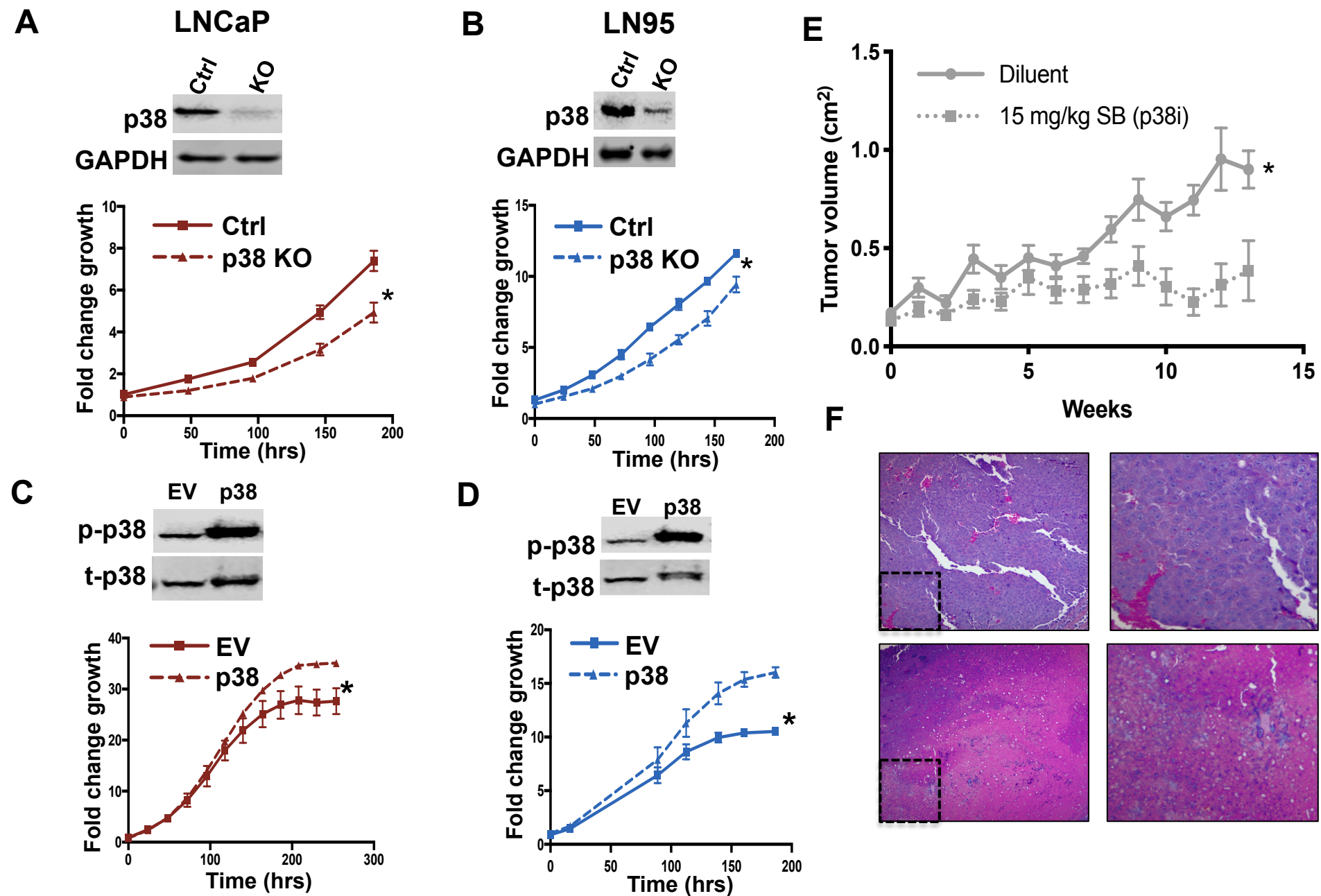
**E**

Cell Line	Prior ADT	expression				genomic				
		AR	AR-V7	GR	NEPC signature	F876L	AR gain	RB loss	TP53 loss	BRCA2 loss
LNCaP EnzaR	-	+	-	-	-	+	-	-	-	-
CS2 EnzaR	+	+	-	+	-	-	-	+	-	+
LN95 EnzaR	+	+	+	-	-	-	-	-	-	-

**Figure 3****A** Up in resistant Down in resistant

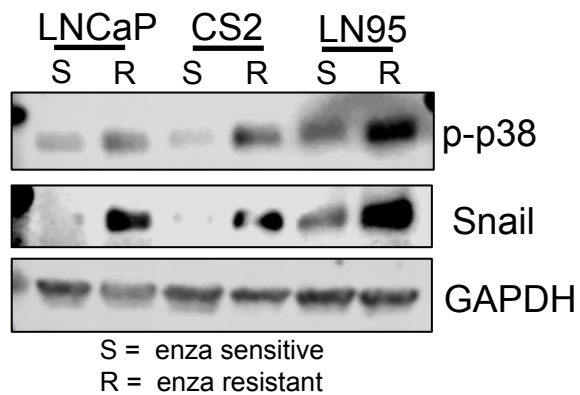


**Figure 4**

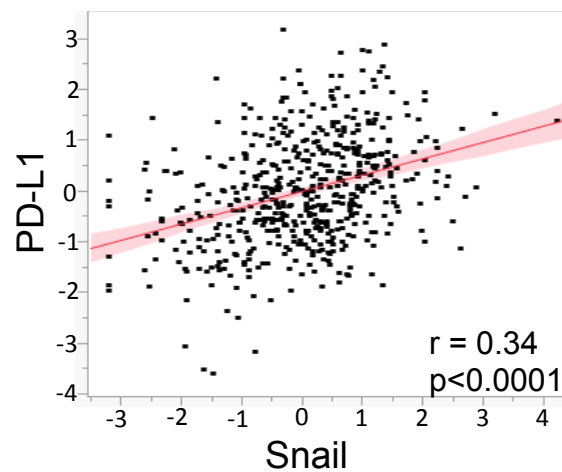
**Figure 5**

**Figure 6**

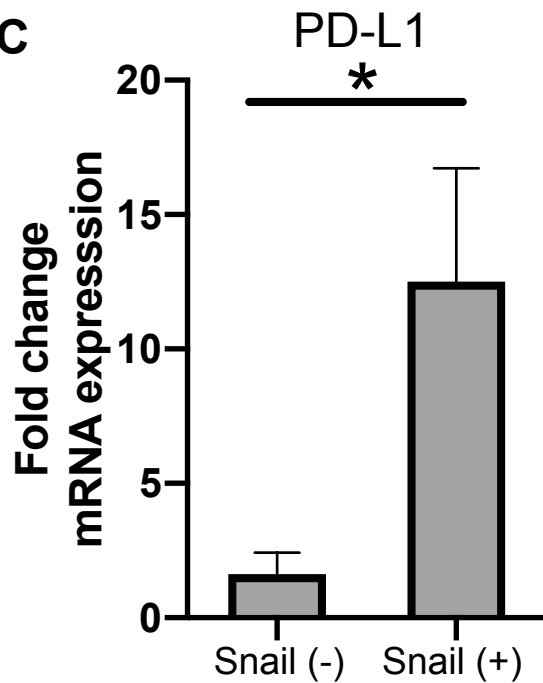
**A**



**B**



**C**



**D**

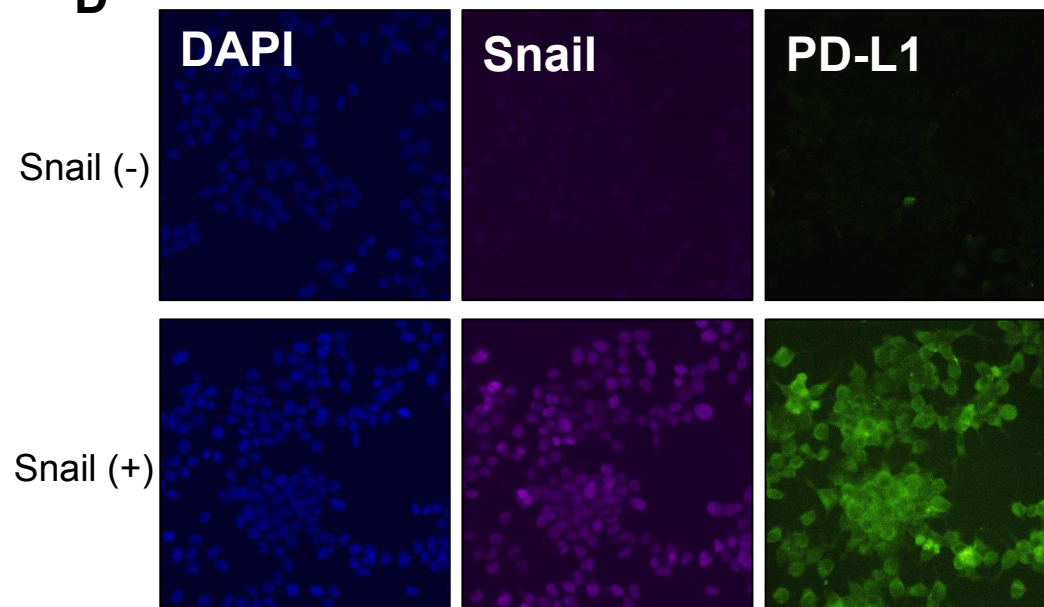


Figure 7

