Checkpoint kinase 2 regulates prostate cancer cell growth through physical interactions with the androgen receptor

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Running title: CHK2 and AR interact to regulate PCa

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ABSTRACT

We have previously demonstrated that CHK2 is a critical negative regulator of AR transcriptional activity, PCa cell growth, and androgen sensitivity. We have now uncovered that the AR directly interacts with CHK2, and ionizing radiation (IR) increases this interaction, which crests one hour after IR-induced DNA damage. This IR-induced increase in CHK2–AR interactions requires AR phosphorylation on both serine81 and serine308 and CHK2 kinase activity. Kinase-impaired CHK2 variants, including the K373E variant associated with 4.2% of PCa, blocked IR-induced CHK2–AR interactions. The destabilization of CHK2-AR interactions induced by the CHK2 variants impairs CHK2 function as a negative regulator of cell growth. CHK2 depletion in LNCaP cells increases transcription of DNAPK and RAD54 and increases clonogenic survival. The data support a model where CHK2 sequesters the AR through direct binding which in turn decreases AR transcription and leads to suppression of PCa cell growth.

32 **INTRODUCTION**

33 Mammalian cells are continuously being bombarded by endogenous and
34 exogenous forces that jeopardize the integrity of DNA. In response to DNA damage, a
35 conserved network of signaling pathways known as the DNA damage response (DDR) is
36 activated to maintain cell viability and genome stability [1]. Prostate cancer (PCa) remains
37 one of the leading causes of death among men of all races (cdc.gov), as castration-
38 resistant prostate cancer (CRPC) is currently incurable. Recently, the DDR has been a
39 focus of PCa research since the androgen receptor (AR), a major driver of PCa,
40 modulates the transcription of DDR genes [2] and DNA repair [3]. We have previously
41 shown that checkpoint kinase 2 (CHK2) negatively regulates androgen sensitivity and
42 PCa cell growth [4].

43 CHK2 is a serine/threonine protein kinase that plays a crucial role in sensing DNA
44 damage and initiating the DDR, comprising of cell cycle arrest, DNA repair, and apoptosis
45 [5]. CHK2 consists of an amino-terminal SQ/TQ cluster domain (SCD) where threonine
46 68 serves as a substrate for phosphorylation by ataxia-telangectasia mutated (ATM)
47 kinase [6]; a carboxy-terminal kinase domain (KD) and nuclear localization sequence [7];
48 and a central forkhead-associated domain (FHA) that provides an interface for
49 interactions with phosphorylated proteins [8]. Currently, there are approximately 24 CHK2
50 substrates in human cells that have been experimentally validated, including polo-like
51 kinase 1 (PLK1), promyelocytic leukemia protein (PML), E2F1, p53, and cell division cycle
52 25C (CDC25C) [9]. These studies show that one mechanism CHK2 utilizes to affect
53 cellular function is through direct protein-protein interactions.

54 For example, the association of CHK2 with PLK1 leads to its localization at
55 centrosomes where it regulates mitotic entry [10]. CHK2 autophosphorylation and
56 activation are regulated by the tumor suppressor PML within PML-nuclear bodies, which
57 are nuclear matrix-associated structures [11]. Binding to PML keeps CHK2 in an inactive
58 state within these PML-nuclear bodies. In return, activated CHK2 can phosphorylate PML
59 on S117 and induce PML-mediated apoptosis. CHK2 can also modify the transcription of
60 apoptotic genes through binding and S364 phosphorylation of the E2F1 transcription
61 factor in response to DNA damage, which stabilizes E2F1 and activates gene
62 transcription [12]. Another way that CHK2 regulates apoptosis is through p53
63 phosphorylation, resulting in the promotion of p53-mediated cell death [13]. The
64 interaction with the core domain of p53 induces an allosteric change in CHK2 which
65 permits p53 S20 phosphorylation [14]. Moreover, CHK2 modulates CDC25C localization
66 by associating with and phosphorylating CDC25 on S216, which creates a binding site
67 for 14-3-3 proteins [15]. 14-3-3 proteins in turn sequester CDC25C in the cytoplasm and
68 block the G2/M transition since cyclin dependent kinase 1 (CDK1) cannot be activated.
69 Finally, our group has shown that CHK2 co-immunoprecipitated with AR in PCa cells and
70 regulated growth, suggesting that AR may be a novel substrate of CHK2 [4]. Thus, given
71 the importance of CHK2 and AR to the DDR and prostate cancer, a full understanding of
72 the functional consequences of the CHK2–AR interaction is required, with the hope of
73 possible clinical applications towards CRPC.

74 Here, we uncovered novel molecular interactions between CHK2 and AR that
75 provide mechanistic insight into our observation that CHK2 negatively regulates prostate
76 cancer growth. We demonstrate that AR directly binds CHK2, and that this interaction

77 increases with ionizing radiation (IR) peaking one hour following exposure. The IR-
78 induced increase in CHK2–AR interaction requires AR phosphorylation on both serine 81
79 and serine 308. The binding of CHK2 with AR is disrupted with CHK2 kinase inhibitors,
80 suggesting that the kinase activity of CHK2 is required for the IR-induced CHK2–AR
81 interaction. This was verified using kinase-impaired CHK2 variants, including the K373E
82 variant associated with 4.2% of PCa. Furthermore, these CHK2 variants exhibit
83 diminished effect on prostate cancer cell growth. Interestingly, CHK2 depletion in LNCaP
84 cells increase transcription of DNAPK and RAD54, as well as clonogenic survival,
85 following IR while decreasing radiation-induced DNA damage repair.
86

87 **RESULTS**

88 **CHK2 directly binds AR**

89 We previously showed that AR coimmunoprecipitated with CHK2 immune
90 complexes in several prostate cancer cell lines [4]. To determine whether this co-
91 association was through direct protein-protein interaction, we performed far western
92 blotting. To generate purified protein for the far westerns, 293T cells were transfected
93 with mammalian plasmids expressing Flag-wtAR, Flag-ERK2, or V5-wtCHK2 (Fig. 1A).
94 We used Flag-ERK2 as a positive control since it has been reported that CHK2 physically
95 associated with ERK1/2 in cancer cells [16]. Flag-ERK and Flag-wtAR targets were
96 immunoaffinity purified and resolved by SDS-PAGE. The target proteins (AR and ERK)
97 on the membrane were probed with purified V5-wtCHK2 protein, crosslinked, and stained
98 with V5 antibodies to detect bound V5-wtCHK2. Membranes were also immunoblotted
99 with AR and ERK1/2 antibodies to confirm that the molecular weight of AR and ERK1/2
100 corresponded with the CHK2 signal, which then indicates direct protein-protein
101 interaction. We found that V5-wtCHK2 bound to Flag-wtAR, as well as the control Flag-
102 ERK2. Moreover, we observed similar results when we performed the converse
103 experiment and observed that the HA-wtAR probe directly associated with the target
104 protein Flag-wtCHK2 (Fig. 1B). These data indicate that the interaction of AR with CHK2
105 is authentic and direct.

106 **Radiation increases CHK2-AR association**

107 Since IR is a standard of care for patients with localized advanced prostate cancer
108 and CHK2 is a known mediator of the DDR, we wanted to assess the impact of IR on
109 CHK2–AR interactions. To examine the effect of radiation on the binding of AR to CHK2,

110 hormone-sensitive LNCaP and castration-resistant Rv1 cells were exposed to 6Gy IR,
111 which is representative of the fractionated dose of IR prostate cancer patients receive
112 [17]. CHK2 immune complexes were generated one hour following radiation. The AR
113 signal was quantified and normalized to total CHK2 protein (Fig. 2). IR significantly
114 increased endogenous CHK2-AR immune complexes by 2-fold and 1.8-fold in LNCaP
115 (Fig. 2A) and Rv1 (Fig. 2B) cells, respectively. Rv1 cells also express AR variant 7 (AR^{V7}),
116 which is a truncated isoform of AR that lacks the ligand binding domain (LBD) [18].
117 Interestingly, endogenous AR^{V7} also bound endogenous CHK2, and IR induced a similar
118 increase in CHK2 – AR^{V7} complexes as that observed with full length AR. Thus, these
119 results suggest that IR drives CHK2 to bind both full length and variant AR.

120 To further characterize the effect of radiation on CHK2-AR associations, we
121 evaluated the kinetics of the increase in CHK2–AR protein complexes in response to IR.
122 CHK2 was immunoprecipitated from irradiated LNCaP and C4-2 cells 1, 4, and 24 hours
123 following IR exposure (Fig. 2C). We found that AR was maximally bound to CHK2 one
124 hour after radiation treatment in both cell lines. This interaction was dramatically reduced
125 to near baseline levels by 4hrs, and returned to baseline levels 24hrs after IR.
126 Interestingly, we noticed that the temporal protein binding of CHK2 to AR paralleled the
127 activation state of CHK2, as represented by CHK2 phosphorylation on threonine 68 (Fig.
128 2C) [Matsuoka, 1998]. Thus, these data show that the CHK2–AR protein complexes are
129 transient, cresting one hour following IR. Furthermore, these observations suggest that
130 the interaction between CHK2 and AR may be regulated by the activation state of CHK2.

131 **AR phosphorylation regulates CHK2–AR interaction**

132 The phosphorylation of AR on serine 81 (S81) by CDK1 and CDK9 stimulates AR
133 transcriptional activity and growth of PCa cells [19]–[21]. Whereas, AR phosphorylation
134 on serine 308 (S308) represses transcription and proliferation and alters AR localization
135 during mitosis [22], [23]. To address whether S81 or S308 phosphorylation influences
136 CHK2–AR complexes, LNCaP cells were transduced with lentiviral particles containing
137 wtAR or AR mutants S81A or S308A (Fig. 3A). The association of AR was analyzed from
138 endogenous CHK2 immune complexes generated one hour after irradiation. There was
139 a 4-fold increase in AR co-immunoprecipitating with CHK2 after IR treatment of cells
140 expressing wtAR. However, neither S81A nor S308A increased in association with CHK2
141 upon IR treatment indicating that phosphorylation of AR on S81 and S308 are required
142 for the IR-induced increase in the interaction between CHK2 and AR.

143 The requirement for irradiation of AR expressing target cells and AR
144 phosphorylation for the IR-induced increase in CHK2–AR association led us to test if AR
145 phosphorylation on S81 and S308 were increased in response to IR. AR was
146 immunoprecipitated from irradiated cells, and phospho-S81 and phospho-S308 were
147 measured by western blotting using phospho-specific antibodies to those sites [20], [24]
148 (Supplemental Figure 1). There were no significant consistent changes in S81 or S308
149 phosphorylation in response to radiation in either cell line. Thus, these findings indicate
150 that while the intensity of S81 and S308 phosphorylation does not markedly change with
151 IR, S81 and S308 phosphorylation is required for CHK2–AR interactions.

152 **CHK2 kinase activity is required for CHK2–AR interaction**

153 To determine if CHK2 kinase activity was necessary for the CHK2–AR association,
154 we tested if CHK2 mutants that are found in PCa and have impaired kinase activity could

155 interact with the AR, and if that interaction increased with IR. We expressed Flag-
156 wtCHK2, Flag-K373E, or Flag-T387N in combination with HA-wtAR in LNCaP cells. The
157 K373E CHK2 mutation impairs CHK2 function suppressing cell growth and promoting
158 survival in response to IR, as a result of reduced kinase activity due to the disruption of
159 CHK2 autophosphorylation [25]. Less is known about the heterozygous missense
160 mutation T387N, but it is reported to diminish kinase activity, and thus, CHK2 function
161 [26]. Flag-CHK2 immunoprecipitations were performed and HA-AR association was
162 evaluated (Fig. 3B). In response to IR there was a striking increase in CHK2–AR co-
163 association in cells expressing Flag-wtCHK2 and HA-wtAR. However, the amount of IR-
164 induced increase in CHK2–AR association in cells expressing either K373E or T387N
165 was dramatically reduced. Therefore, these data indicate that the kinase activity of CHK2
166 is required for the interactions of CHK2 and AR, and that the CHK2 mutant associated
167 with PCa, T387N, has a diminished ability to interact with the AR.

168 **IR increases direct CHK2–AR binding**

169 To both confirm that IR induces the increase of CHK2–AR protein complexes and
170 determine if the increase is due to direct protein-protein interaction, we carried out far
171 western blotting where target (Flag-wtAR and Flag-ERK2) or probe proteins (V5-wtCHK2)
172 were isolated from cells either irradiated with 6Gy 48hrs following transient transfection,
173 and purified one hour after radiation exposure, or kept untreated prior to immunoaffinity
174 purification of target and probe proteins. When the cells expressing the V5-wtCHK2 probe
175 and Flag-wtAR target were irradiated there was a 2-fold increase in V5-wtCHK2 binding
176 to the target Flag-wtAR in response to IR (Fig. 4A). However, when the V5-wtCHK2 probe
177 was not treated with IR, no increase in probe binding to target was observed (Fig. 4B).

178 Together, these data indicate that radiation of cells expressing the V5-wtCHK2 probe is
179 required for the increased CHK2–AR interaction that occurs in response to IR. Since IR
180 increases the activity state of CHK2 as measured by phosphorylation of CHK2, this result
181 supports the above data indicating that CHK2 kinase activity is required for the IR induced
182 CHK2–AR association.

183 **AR and CHK2 activity required for direct CHK2-AR binding**

184 To determine whether AR activity is required for the increase in CHK2–AR direct
185 binding in response to IR, far westerns were again performed by treating 293T cells
186 expressing Flag-wtAR, Flag-ERK2, or V5-wtCHK2 with 6Gy ionizing radiation in the
187 presence or absence of the anti-androgen Enzalutamide (Fig. 5A). As expected, far
188 western blots revealed that radiation increased purified CHK2 binding to purified AR by
189 2-fold. The presence of enzalutamide blocked the IR induced increase in CHK2–AR
190 interaction, suggesting that transcriptionally activated AR is required for the IR-induced
191 increase in association of CHK2 and AR. This is consistent with the loss of AR
192 phosphorylation on S81 decreasing the CHK2–AR association.

193 CHK2 activity is also required for the direct CHK2–AR binding in response to IR.
194 In parallel to the far western in Fig 5A, we performed far westerns with the cells expressing
195 the V5-CHK2 probe pre-treated with the CHK2 inhibitor BML-277 one hour prior to IR.
196 (Fig. 5B). Remarkably, inhibition of CHK2 kinase activity completely blocked the increase
197 in CHK2–AR interactions that we observed when the probe was irradiated in the absence
198 of BML-277 (Fig. 5A). We also did not detect increased binding to the control target Flag-
199 ERK2. These data confirm the lack of increased CHK2–AR binding when cells the V5-
200 CHK2 probe was isolated from were not irradiated (Fig. 4B). These results, along with the

201 data in Figure 3B, indicate that the direct CHK2–AR protein binding requires CHK2 kinase
202 activity.

203 **PCa CHK2 mutants limit suppression of PCa growth**

204 Since CHK2 regulates the cell cycle and PCa cell growth [4], [14], we investigated
205 the effect of CHK2 PCa mutations, which disrupt the CHK2–AR interaction, on CHK2
206 regulation of PCa cell growth (Fig. 6). PCa cells were transduced with vector or CHK2
207 shRNA lentivirus to deplete endogenous CHK2, and then CHK2 expression was rescued
208 with wtCHK2, K373E, or T387N. Cell growth was quantitated in the presence and
209 absence of 0.05nM synthetic androgen R1881 seven days following transduction using
210 CyQUANT, which assesses cell proliferation as a function of DNA content. In agreement
211 with previous reports [4], hormone stimulated growth of vector-expressing cells and
212 knockdown of CHK2 significantly augmented growth in all PCa cell lines tested. Re-
213 expression of wtCHK2, K373E, and T387N in CHK2-depleted cells markedly suppressed
214 the increase in growth induced by CHK2 knockdown in all PCa cell lines tested.
215 Interestingly, in LNCaP cells the extent of growth inhibition induced by K373E and T387N
216 was significantly less than that generated by wtCHK2 (Fig. 6A). The effect of CHK2 re-
217 expression on cell growth in castration-resistant C4-2 (Fig. 6B) and Rv1 (Fig. 6C) cells
218 was not significantly different between wtCHK2 and the kinase deficient K373E and
219 T387N mutants, although the magnitude of inhibition caused by the mutants was
220 consistently less than that produced by wtCHK2. Expression levels of wtCHK2, K373E,
221 and T387N do not account for the difference observed between LNCaP and C4-2 or Rv1
222 (Fig. 6) since the relative expression of wtCHK2, K373E, and T387N were similar across
223 the cell lines. These observations suggest that CHK2 kinase activity, which is reduced in

224 the PCa mutant K373E, limits the ability of CHK2 to negatively regulate PCa cell growth,
225 especially in androgen dependent PCa. This then raises the possibly that the PCa CHK2
226 K373E mutant with diminished AR binding is selected for decreasing CHK2 suppression
227 of AR activity and PCa cell growth.

228 **CHK2 suppresses IR induction of DNAPK and RAD54**

229 Reports in the literature suggest that the AR is a critical regulator of genes in the
230 DDR [2], [3]. Therefore we evaluated the impact of CHK2 knockdown on IR induced
231 transcription of DDR genes in LNCaP cells (Fig. 7). In our experiments, androgen and IR
232 only affected DNAPK and RAD54 transcript levels; we did not observe androgen or IR
233 induction of XRCC2, XRCC3, XRCC4, XRCC5, MRE11, RAD51, FANC1 and BRCA1
234 transcripts as reported by others (data not shown) [2], [3]. This discrepancy is consistent
235 with the observation that androgen regulation of DDR genes is specific to the model
236 system and disease state examined [27]. Knockdown of CHK2 in LNCaP cells grown in
237 CSS and stimulated with 1nM DHT led to an increase in transcription of DNAPK and
238 RAD54. This increase was further augmented by IR suggesting that CHK2 may suppress
239 AR transcription of DDR genes in response to IR.

240 **CHK2 effect on IR sensitivity and DNA repair**

241 We next examined the impact of CHK2 interactions on cell survival and sensitivity
242 to IR (Fig. 8A). Increasing doses of IR were delivered to LNCaP cells expressing CHK2
243 (pLKO) or depleted of CHK2 (CHK2 KD). Cells were seeded and allowed to grow for 14
244 days. Clonogenic assays revealed that CHK2 knockdown promoted cell survival following
245 ionizing radiation. This data, along with the data above indicating that CHK2 suppresses

246 AR transcription of DNA repair genes suggested the hypothesis that loss of CHK2 could
247 facilitate DNA repair.

248 To assess the effect of IR-induced DNA damage in the presence or absence of
249 CHK2 knockdown we performed comet assays, which measures DNA breaks [28], and
250 immunofluorescence staining of phospho-γH2AX, a marker for DNA double-strand breaks
251 [29], [30]. Since the majority of IR induced DSBs are repaired rapidly [29], [31] we focused
252 on early timepoints following DNA damage. Neither LNCaP or Rv1 cells showed a change
253 in IR induced comet tail moment following 1 hour of IR (Supplemental Figure 2). In order
254 to quantify phospho-γH2AX foci in an unbiased manner we developed an approach that
255 utilizes automated quantitation of immunofluorescence that enables us to rapidly
256 determine the signal intensity (Supplemental Figure 3). LNCaP cells were transduced
257 with vector or CHK2 shRNA 48hrs before delivery of IR. Radiation induced similar levels
258 of DNA damage 15min after exposure regardless of CHK2 expression (Fig.8B,C).
259 However, CHK2 knockdown exhibited a significant increase in phospho-γH2AX signal
260 compared to vector control cells 45min after IR. γH2AX is phosphorylated in response to
261 inputs in addition to IR induced DNA double strand breaks including RNA polymerase II
262 dependent transcription [32]–[34]. Thus, the apparent discrepancy in the comet and
263 γH2AX foci assay may be explained by the increase in AR transcription when CHK2 is
264 knocked down as in Figure 7 and in our previous study [4].

265 Since superphysiological doses of androgen has been associated with
266 transcription dependent double strand breaks [35], [36], we tested if CHK2 knockdown
267 would augment superphysiological androgen dependent phospho-γH2AX foci. LNCaP
268 and Rv1 cells were transduced with vector or CHK2 shRNA 48hrs before treatment with

269 a range of the synthetic androgen R1881 up to 100nM for 6 hours (Supplemental Figure
270 4). We saw modest hormone induced phospho-γH2AX foci and no change with CHK2
271 knockdown. The superphysiologic dose of androgen likely maximally activates AR
272 dependent transcription negating the increase in AR transcriptional activity typically
273 observed with CHK2 knockdown.
274

275 **DISCUSSION**

276 PCa is the most frequently diagnosed cancer and the second leading cause of
277 cancer death among American men, with approximately 88 men dying from PCa every
278 day (pcf.org). While androgen deprivation therapy (ADT) is effective initially, most patients
279 will relapse and develop incurable CRPC. Recently, there has been an emphasis on
280 understanding the link between the DDR and AR, since radiation is a standard of care for
281 locally advanced PCa where the AR is a major driver, and PARP inhibitors may be
282 efficacious in CRPC patients with mutations in DDR genes [2]–[4], [37]–[39].

283 Our study identifies AR as a direct interacting protein with CHK2 in PCa cells.
284 Several studies elucidated the role of DDR protein-AR interactions in modulating AR
285 transcriptional activity. PARP-1 was recruited to AR binding sites, enhancing AR
286 occupancy and transcriptional function [40]. Tandem mass spectroscopy analysis
287 identified Ku70 and Ku80 as direct AR-interacting proteins that positively regulate AR
288 transactivation [41]. Furthermore, BRCA1 physically interacted with the DNA-binding
289 domain (DBD) of AR to enhance AR transactivation and induce androgen-mediated cell
290 death through p21 expression [42]. In contrast, the association of the LBD of AR with
291 hRad9, a crucial member of the checkpoint Rad family, suppressed AR transactivation by
292 preventing the androgen-induced interaction between the n-terminus and c-terminus of
293 AR [43]. Other groups reported non-genomic effects as a result of DDR protein-AR
294 interactions. Mediator of DNA damage checkpoint protein 1 (MDC1), an essential player
295 in the Intra-S phase and G2/M checkpoints, physically associated with FL-AR and AR^{V7}
296 to negatively regulate PCa cell growth and migration [44]. Yin and colleagues, on the
297 other hand, showed that increased clonogenic survival following IR was a consequence

298 of DNA-PKc directly complexing with both FL-AR and AR^{V5-7}, with radiation increasing
299 these interactions and enzalutamide blocking the association with FL-AR but not AR^{V5-7}
300 [45]. These data support a model where AR is integrated in the DDR, interfacing at
301 multiple points in the DDR.

302 We show that the association of CHK2 and AR requires phosphorylation of AR on
303 S81 and S308. Since proteins containing FHA domains bind phosphoproteins [8], we
304 hypothesize that AR interacts with CHK2 through the CHK2 FHA domain. In support of
305 this, the Zhao lab determined that AR physically associated with the FHA domain of
306 another critical DDR member, MDC1 [44]. Expression of truncation mutants of different
307 MDC1 domains in LNCaP cells led to the discovery that AR only co-immunoprecipitated
308 with MDC1 mutants containing the FHA domain in the absence and presence of
309 dihydrotestosterone. Their results indicated that the FHA domain of MDC1 mediated the
310 interaction with AR. Here we report that AR phosphorylation on S81 and S308 is required
311 for CHK2–AR binding. Interestingly neither of these phosphorylation sites were altered by
312 IR. AR S81 and S308 can both be phosphorylated by CDK1, which is downstream of
313 canonical CHK2 signaling [21], [24], and was the motivation for us examining these sites
314 in response to IR. The prediction is that IR would lead to a decrease in S81 and S308
315 phosphorylation. However, our previous studies demonstrated that S81 is predominantly
316 phosphorylated by CDK9, and thus is more indicative of AR transcriptional activity [20].
317 We also found that S308 phosphorylation was restricted to late G2 and M phase of the
318 cell cycle [24]. CDK9 phosphorylation of S81 and the restriction of S308 phosphorylation
319 to G2/M likely accounts for not observing significant changes in these phosphorylation
320 sites in response to IR. The disconnect between these sites being required for the IR

321 induced increase in CHK2–AR association but not being regulated by IR suggests that
322 the hormone induced activation state of the AR is a critical determinant in the IR induced
323 increase in CHK2–AR association.

324 In our experiments examining CHK2–AR binding, we used ERK as a positive
325 control for a protein that interacts with CHK2 [16]. Interestingly, we observed a significant
326 increase in CHK2–ERK association with IR. This is consistent with IR increasing CHK2
327 T68 phosphorylation, which is required for the CHK2–ERK interaction. These data point
328 to a potential larger role for CHK2 beyond canonical DDR and cell cycle checkpoint
329 signaling; consistent with this notion CHK2 has been implicated in diverse cellular
330 processes [46]–[48]. MEK inhibition is effective in lung tumors with ATM mutations where
331 CHK2 is inactive [49] providing further support that CHK2 negatively regulates ERK.
332 CHK2 negatively regulating both the AR and ERK suggests the hypothesis that CHK2
333 may serve as a general negative regulator of mitogenic signals in response to IR.

334 We found that CHK2 variants with diminished kinase activity impaired the IR-
335 induced increase in CHK2–AR interaction but did not completely block the CHK2–AR
336 interaction. This correlated with a reduced inhibition of cell growth by the CHK2 variants.
337 CHK2-depleted cells re-expressing CHK2 variants exhibited an approximate 2-3-fold
338 reduction in growth inhibition in response to hormone when compared to cells re-
339 expressing wtCHK2. Moreover, the fold change in suppression of growth between
340 wtCHK2 and CHK2 variants was greater in androgen-dependent LNCaP cells than in
341 castration-resistant C4-2 and Rv1 cells, suggesting that in hormone sensitive PCa CHK2
342 variants may play a larger role in regulating growth. Berge and colleagues discovered
343 numerous CHK2 splice variants in breast cancer tissue, where all variants were co-

344 expressed with wtCHK2 [50]. Furthermore, several of these variants reduced kinase
345 activity when simultaneously expressed with wtCHK2 and displayed a dominant negative
346 effect on wtCHK2. The impact of the CHK2 variants found in PCa on wtCHK2 function
347 has not yet been fully explored.

348 Multiple studies indicate that the AR is a critical regulator of genes in the DDR [2],
349 [3]. Reports by others demonstrate that androgen and IR increased DNAPK, XRCC2, and
350 XRCC3 [3]. This concept was supported by more global analysis of transcripts
351 demonstrating androgen regulation of DDR genes [2]. Our data indicating that CHK2
352 knockdown increases DNAPK and RAD54 transcript levels leads to the hypothesis that
353 CHK2 binding to the AR suppresses AR transcription of DDR genes enabling cells to turn
354 off the DDR following DNA repair. This is consistent with our earlier observation that
355 CHK2 knockdown led to the increase in the transcripts of canonical AR target genes [4].

356 We observed radiation resistance in CHK2 knockdown cells, consistent with CHK2
357 suppression of AR transcription of DDR genes. We also observed an increase in
358 phospho-γH2AX signal when CHK2 was knocked down, but no change in DNA breaks as
359 measured by the comet assay under similar conditions. These paradoxical results may
360 be explained by phosphorylation of γH2AX in response to transcription induced DNA
361 breaks [32]–[34]. These incongruous results may also be due to competing effects of
362 CHK2 as both a regulator of the cell cycle and apoptosis [46].

363 The data reported herein along with our previous work [4] indicate that CHK2 acts
364 as a tumor suppressor in PCa, either through loss of expression or mutation. This raises
365 the concern that CHK2 antagonists in clinical development may paradoxically lead to
366 enhanced PCa growth and resistance to IR. However, it is important to note that we have

367 predominately used a RNAi/overexpression approach. Our RNAi approach is more similar
368 to the CHK2 variants in PCa that have reduced kinase activity. It is important to consider
369 that pharmacologic inhibition is different than inhibition by RNAi [51]. A pharmacologic
370 approach that provides a sudden and complete inhibition of CHK2 kinase activity may
371 impact PCa differently than our RNAi approach, especially when combined with IR or AR
372 antagonists. Our work and that in the literature also suggests that approaches
373 downstream of CHK2 may be more straightforward than targeting CHK2.

374 In this study, we presented data that provides mechanistic insight into our
375 observation that CHK2 negatively regulates PCa growth. We demonstrated that AR
376 directly bound CHK2, and that IR elevated the CHK2–AR interaction, which peaked one
377 hour following exposure. Not only did these CHK2–AR protein complexes require AR
378 phosphorylation on both serine 81 and serine 308, but CHK2 kinase activity was also
379 necessary, as CHK2 kinase inhibitors disrupted CHK2–AR binding. This was verified
380 using kinase-impaired CHK2 variants, including the K373E variant associated with 4.2%
381 of prostate cancer. Furthermore, these CHK2 variants exhibited a diminished effect on
382 restricting prostate cancer cell growth. We observed that knockdown of CHK2 led to an
383 increase in PCa cell survival in response to IR. This suggests that the deregulation of
384 CHK2 in PCa compromises the DDR and can confer resistance to radiation. In a previous
385 study, we showed that CHK2 knockdown hypersensitized PCa cells to castrate levels of
386 androgen and increased AR transcriptional activity on both androgen-activated and
387 androgen-repressed genes [4]. As part of a feedback loop, AR transcriptionally represses
388 CHK2 levels. Thus, these data along with our previously published results suggest a
389 model where CHK2 antagonizes AR through direct binding and inhibition of transcription

390 of AR targets, including DDR genes (Figure 9). The K373E mutation of CHK2 or loss of
391 CHK2 expression in PCa leads to increased AR transcriptional activity and survival in
392 response to DNA damage, all leading to a more aggressive cancer. Collectively, the work
393 provides a foundation for the continued study of CHK2–AR interactions and functional
394 consequences to benefit PCa therapies.
395

396 **MATERIALS AND METHODS**

397 **Cell culture**

398 LNCaP and C4-2 cells (a gift from Dr. L. W. K. Chung) were grown in DMEM:F12
399 (Invitrogen) with 5% Non-Heat Inactivated serum (Gemini) and 1% Insulin-Transferrin-
400 Selenium-Ethanolamine (ITS) (ThermoFisher). CWR22Rv1 (Rv1) (gift from Drs. Steven
401 Balk) and 293T cells (gift from Dr. Tim Bender) were grown in DMEM (Invitrogen) with
402 10% Heat-Inactivated serum. For growth experiments, phenol-red free DMEM:F12 media
403 with 5% Charcoal-Stripped Serum (CSS) (Gemini) was used. Commercial DNA
404 fingerprinting kits (DDC Medical) verified cell lines. The following STR markers were
405 tested: CSF1PO, TPOX, TH01, Amelogenin, vWA, D16S539, D7S820, D13S317 and
406 D5S818. Allelic score data revealed a pattern related to the scores reported by the ATCC,
407 and consistent with their presumptive identity.

408 **Reagents**

409 Transfection: Fugene 6 (Promega); TransIT-2020 (Mirus Bio).Inhibitors: Enzalutamide
410 (Selleck Chemicals), BML-277 (Santa Cruz Biotech).Antibodies: CHK2 (2G1D5), pCHK2
411 T68, ERK1/2 (137F5), Actin, Flag-Tag, V5-Tag, HA-Tag, γH2AX (Cell Signaling); AR, pAR
412 S308 (in-house); pAR S81 (Millipore); Cy3-labeled donkey anti-rabbit (Jackson
413 ImmunoResearch). Western blotting performed as previously described [4].

414 **Far Western Blot**

415 To measure direct protein interactions, the protocol was adapted from Prickett *et*
416 *al* [52] and Wu *et al* [53]. 293T cells were transfected with 1) Flag-wtAR, 2) HA-wtAR, 3)
417 Flag-ERK2, 4) V5-wtCHK2, or 5) empty vector control. Cells were treated with vehicle,
418 enzalutamide, or BML-277, one hour before ionizing radiation (IR) exposure. Whole cell

419 extracts were made using Triton-X lysis buffer, sonicated, and immunopurified using anti-
420 Flag, anti-HA, or anti-V5 beads (Sigma) for 2hr at 4°C. Protein bound to beads was
421 washed three times with Triton-X lysis buffer, eluted with 35μl 2X sample buffer, and
422 boiled for 5 min. Proteins were resolved by SDS-PAGE and transferred to PVDF
423 membrane. Proteins on the membrane were denatured and renatured in buffers with
424 varying guanidine–HCl concentrations. Membranes were blocked in 3% blocking buffer
425 (3% bovine serum albumin in Tris-buffered saline/Tween 20) for 1hr. Probes were diluted
426 in 3% blocking buffer and incubated overnight at 4°C. Membranes were washed three
427 times with PBS for 5min followed by fixation using 0.5% paraformaldehyde for 30 min at
428 room temperature. Membranes were then rinsed quickly twice with PBS and quenched
429 using 2% glycine in PBS for 10 min at room temperature. The membrane was blotted for
430 Flag, HA, V5, AR, CHK2, or ERK1/2 and analyzed using the LI-COR Odyssey system
431 and software.

432 **Immunoprecipitation**

433 CHK2 or AR protein was immunoprecipitated from 1mg cell lysate from LNCaP,
434 C42, and Rv1 cells cultured in the appropriate growth media or LNCaP cells transiently
435 transfected with Flag-wtAR/Flag-S81A/Flag-S308A plus V5-wtCHK2 or Flag-
436 wtCHK2/Flag-K373E/Flag-T387N plus HA-wtAR for 48hrs; treated with radiation.
437 Immunoprecipitations were performed with either agarose or magnetic beads, proteins
438 were separated by 7.5% SDS-PAGE; and immunoblotted with AR, pAR S81, pAR S308,
439 CHK2, pCHK2 T68, HA, or ERK1/2 antibodies.

440 **CyQuant Growth Assays**

441 Assay was performed as previously described [4]. Briefly, shCHK2-209, shCHK2-
442 588, wtCHK2, K373E, T387N or Vector control virus was added to fibronectin-coated
443 (1μg/ml) 96well plates. Constructs of CHK2 wild-type and variants were verified by
444 sequencing. Cells were plated in phenol-red free DMEM:F12 or DMEM media with 5%
445 CSS in the presence or absence of 0.05nM R1881. CyQuant reagent was added on Day
446 7 according to the manufacturer's protocol (ThermoFisher). Quantification was performed
447 using a BioTek Synergy 2 plate reader.

448 **qPCR**

449 RNA isolation and quantitative real-time PCR (qPCR) was performed as previously
450 described [54], [55]. RNA concentrations were determined using a NanoDrop 2000 UV-
451 Vis Spectrophotometer (Thermo Scientific). Primer sequences and annealing
452 temperature: DNAPKc FW (ATGAGTACAAGCCCTGAG); DNAPKc RV
453 (ATATCAGAGCGTGAGAGC) (Tm=60deg). RAD54B FW
454 (ATAACAGAGATAATTGCAGTGG); RAD54B RV (GATCTAATGTTGCCAGTGTAG)
455 (Tm=60deg). PSMB6 FW (CAAACTGCACGGCCATGATA); PSMB6 RV
456 (GAGGCATTCACTCCAGACTGG) (Tm=60deg).

457 **Clonogenic Survival Assay**

458 LNCaP cells were transduced with lentiviral particles expressing vector or CHK2
459 shRNAs and treated with 0-6Gy of radiation 72hrs after transduction. Cells were
460 trypsinized, counted, and appropriate numbers were plated in triplicate with the
461 appropriate growth media for colony formation assays (100 cells/0Gy, 200 cells/2Gy,
462 1000 cells/4Gy, and 6000 cells/6Gy). After 10-14 days, colonies consisting of 50-70 cells
463 were counted using crystal violet. Plotted is the surviving fraction (number of colonies

464 counted/(number of cells seeded x PE) where PE = plating efficiency = number of colonies
465 counted/number of cells seeded) following radiation.

466 **Comet Assay**

467 All Comet Assay steps were performed in the dark. Cells were washed twice with
468 PBS , scraped and suspended in PBS. Cells were combined with molten LMAgarose
469 (Trevigen, 4250-050-02) and placed into comet suitable sides. Samples were left at 4°C
470 for 15 minutes order to create flat surface. Slides were immerse in lysis solution (Trevigen,
471 4250-050-01) for 40 minutes at 4°C and then in alkaline solution for 30min at room
472 temperature. Slides were electrophoresed in 200mM NaOH, 1mM EDTA in water; pH>13
473 at 21Volts (300mA) for 30 minutes at 4°C. After the electrophoresis, slides were gently
474 drained, washed twice in dH2O for 10min and immersed in 70% ethanol for 5min.
475 Samples were left to dry overnight at RT. Samples were stained with SYBR Green at 4°C
476 for 5 minutes and left to dry completely overnight. For quantification, images were
477 acquired using a fluorescence microscope (Olympus BX51, High-mag) equipped with a
478 20×, 0.5 NA objective and a camera (DP70). Images were acquired with DPController
479 software. Images were analyzed by ImageJ software and graphs generated using Prism
480 (GraphPad Software). All imaging was performed at ~24°C.

481 **Immunofluorescence (IF)**

482 LNCaP cells were transduced with lentivirus expressing vector or CHK2 shRNAs
483 on 1μg/ml fibronectin-coated coverslips and treated with radiation 48hrs after
484 transduction. Cells were allowed to recover from IR exposure for 0, 15, and 45mins.
485 Coverslips were washed 3X with PBS, permeabilized with 0.2% Triton-X for 10mins,
486 blocked with 2% FBS/BSA/donkey serum in PBS for 2hrs at room temperature, and

487 incubated with γH2AX antibodies overnight at 4°C. Coverslips were mounted with
488 Vectashield containing DAPI (ThermoFisher), and images were acquired with a LSM 880
489 confocal microscope (Carl Zeiss). γH2AX signals were measured using ImageJ software.

490 **Scientific Rigor**

491 Each experiment was performed independently a minimum of three times and
492 each experiment had technical replicates for measuring the endpoint. An independent
493 experiment is defined as an experiment performed on a different day with a different
494 passage number. The number of independent experiments is reported in each figure
495 legend. All data are shown, no outliers were removed. Statistical analysis was performed
496 using GraphPad Prism 8.2.1 and the test used is reported in each figure legend.

497

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COMPETING INTERESTS

The authors declare no competing financial interests

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656

657

658 **FIGURE LEGEND**

659 **Figure 1. CHK2 directly binds AR.** 293T cells were transfected with Vector, Flag-wtAR,
660 Flag-wtCHK2, Flag-ERK2, HA-wtAR, or V5-wtCHK2 using Fugene 6. 48hrs following
661 transfection, Flag, HA, and V5 were immunoprecipitated, and far western blotting was
662 performed. Membranes were blotted with the following antibodies: Flag, HA, V5, AR,
663 CHK2, and ERK2. Blots were visualized using the Odyssey CLx. (A) Probe = V5-wild-
664 type CHK2; Targets = Flag-wtAR and Flag-ERK2. Representative blots are shown, n=3.
665 (B) Probe = HA-wtAR; Targets = Flag-wtCHK2. Representative blots are shown, n=3.
666

667 **Figure 2. Radiation transiently increases CHK2-AR association.** CHK2 immune
668 complexes were generated one hour following radiation (6Gy) from 1mg cell extract from
669 (A) LNCaP and (B) Rv1 cells grown in serum-supplemented media, separated by 7.5%
670 SDS-PAGE, and immunoblotted with AR, CHK2, and ERK1/2 antibodies. Plotted is the
671 AR or AR-V7 signal normalized to total CHK2, and compared to untreated cells (Rv1).
672 Representative blots are shown for (A) LNCaP ($n=4$, $p<0.003$) and (B) Rv1 cells ($n=3$,
673 $p<0.02$). (C,D) LNCaP and C4-2 cells were seeded in serum-supplemented growth media
674 and allowed to adhere for 48hrs. Cells were exposed to 6Gy IR and CHK2 immune
675 complexes were immunoprecipitated using a magnetic bead system 0-24hrs after
676 radiation from 1mg cell extracts, separated by 7.5% SDS-PAGE, and blotted with AR,
677 CHK2, pCHK2 T68, and ERK1/2 antibodies. (C) Representative blots are shown, n=3.
678 (D) Plotted is the AR signal normalized to total CHK2 and compared to untreated cells,
679 $p<0.0001$. Error bars, SEM. Band signals were quantitated on Odyssey LICOR imaging
680 system. Statistical analysis was performed using ANOVA and Tukey test.

681

682 **Figure 3. AR phosphorylation and CHK kinase activity regulates CHK2-AR**
683 **association.** (A) CHK2-AR interactions requires AR phosphorylation on serines 81 and
684 308. LNCaP cells were transduced with lentiviral particles expressing wtAR, S81A, or
685 S308 for 48hrs. Cells were irradiated with 6Gy, and CHK2 was immunoprecipitated one
686 hour after IR. Representative blots are shown. Plotted is the AR signal normalized to total
687 CHK2 and compared to untreated cells, n=3, $p<0.009$. Error bars, SEM. Blots were
688 quantitated on Odyssey LICOR imaging system. Statistical analysis was performed using
689 ANOVA and Tukey test. (B) Expression of CHK2 variants with reduced kinase activity
690 inhibits the radiation-induced increase in CHK2-AR interactions. LNCaP cells were
691 transfected with HA-wtAR, HA-S81A, or HA-S308 in combination with Flag-wtCHK2 for
692 48hrs using TransIT-2020 (Mirus). Cells were irradiated with 6Gy, and Flag was
693 immunoprecipitated using a magnetic bead system one hour after IR. Representative
694 blots are shown. Plotted is the HA-AR signal normalized to total Flag-CHK2 and
695 compared to untreated cells. Error bars, SEM. Blots were quantitated on Odyssey LICOR
696 imaging system. Statistical analysis was performed using ANOVA and Tukey test, n=3,
697 $p<0.02$.

698

699 **Figure 4. Radiation increases direct CHK2-AR binding.** (A) Radiation increases direct
700 binding of AR and CHK2. Probe = V5-wtCHK2 + IR; Targets = Flag-wtAR and Flag-ERK2
701 -/+ IR. Representative blots are shown, n=3, p=0.004. (B) Radiation of both CHK2 and
702 AR is required for the increase in direct association. Probe = V5-wtCHK2 - IR; Targets =
703 Flag-wtAR and Flag-ERK2 -/+ IR. Representative blots are shown, n=3. Quantitation was

704 performed on Odyssey LICOR imaging system. Error bars represent standard error of the
705 mean (SEM). Statistical analysis was performed using ANOVA and Tukey test.
706
707 **Figure 5. AR and CHK2 activity required for direct CHK2-AR binding.** (A)
708 Enzalutamide significantly impairs the association of CHK2 with AR. 293T cells were
709 transfected with Vector, Flag-wtAR, Flag-ERK2, or V5-wtCHK2 using Fugene 6. 48hrs
710 following transfection, cells were irradiated with 6Gy, and Flag and V5 were
711 immunoprecipitated one hour following radiation. Far western blotting was performed.
712 Membrane was blotted with the following antibodies: V5, AR, and ERK2. Representative
713 blots are shown. Plotted is the V5-CHK2 signal normalized to total wtAR or ERK2 and
714 compared to untreated cells, n=3, $p<0.02$. Error bars, SEM. (B) Inhibition of CHK2 with
715 BML-277 blocks the increase in CHK2-AR interactions. 293T cells were transfected with
716 Vector, Flag-wtAR, Flag-ERK2, or V5-wtCHK2 using Fugene 6. 48hrs following
717 transfection, cells were pre-treated with vehicle or 10μM BML-277 for 1hr, irradiated with
718 6Gy, and Flag and V5 were immunoprecipitated one hour following radiation. Far western
719 blotting was performed. Membrane was blotted with the following antibodies: V5, AR, and
720 ERK2. Representative blots are shown. Plotted is the V5-wtCHK2 signal normalized to
721 total AR or ERK2 and compared to untreated cells, n=3. Error bars, SEM. No statistical
722 difference was observed between the groups.
723
724 **Figure 6. Wild-type CHK2 negatively regulates prostate cancer cell growth.** (A)
725 LNCaP, (B) C4-2, and (C) Rv-1 cells were transduced with lentiviral particles expressing
726 vector, shCHK2-exon 12, or shCHK2-3'UTR in combination with wtCHK2, K373E, or

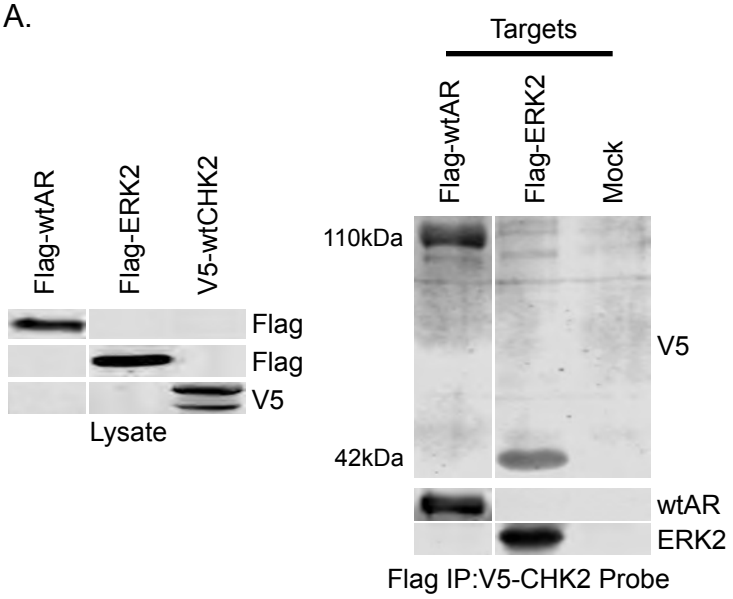
727 T387N in the presence or absence of 0.05nM R1881. CyQuant assay was performed 7
728 days after transduction. Cell growth was compared with untreated vector control and the
729 values were averaged across biological replicates. Error bars, SEM, n=3. Statistical
730 analysis was performed using ANOVA and Tukey test, $p<0.01$. Representative blots of
731 CHK2 expression are shown.
732
733 **Figure 7. CHK2 knockdown increases the transcription of DDR genes in the**
734 **presence and absence of radiation.** Transcript levels of DDR genes in LNCaP cells
735 transduced with CHK2 shRNAs and pLKO vector control and grown in CSS
736 supplemented with 1nM DHT were measured by qPCR. 48hrs following transduction,
737 cells were exposed to 2Gy ionizing radiation and RNA was isolated 6 hours later.
738 Transcript levels were normalized to the housekeeping gene, PSMB6, and compared to
739 pLKO. Values were averaged across biological replicates +/- standard error of the mean,
740 n=3. Shown are the histograms for (A) DNAPKc and (B) Rad54B in LNCaP cells.
741 Statistical analysis was performed using one-way ANOVA and Tukey's test. * $p<0.02$.
742
743 **Figure 8. CHK2-depleted cells show increased survival and DNA damage following**
744 **radiation.** (A) Knockdown of CHK2 desensitizes cells to IR. LNCaP cells were transduced
745 with lentiviral particles expressing pLKO or CHK2 shRNAs for 48hrs, treated with 0-6Gy
746 IR, and seeded at the appropriate cell number for colony survival assays. Results were
747 normalized to untreated pLKO control and fitted to a standard linear quadratic model.
748 Error bars, SEM. Statistical analysis was performed using the Student's t-test, n=4-8,
749 $p<0.01$. (B) Representative images of phospho-γH2AX, CHK2, and AR immunostaining

750 quantified in (C) . (C) phospho-γH2AX is elevated in cells depleted of CHK2. LNCaP cells
751 were transduced with lentiviral particles expressing empty vector or CHK2 shRNAs on
752 fibronectin-coated coverslips in the appropriate growth media. Cells were irradiated with
753 5Gy after 48hrs. Coverslips were processed for IF at 0, 15, and 45min following IR. Plotted
754 is the γH2AX signal, which equals the mean grey value intensity x number of foci per
755 nucleus. Statistical analysis was performed using ANOVA and Tukey test, $p<0.0001$.
756
757 **Figure 9. Model of CHK2-AR.** We hypothesize the following model. In response to IR,
758 CHK2 activation antagonizes AR through direct binding and inhibition of transcription of
759 AR targets. CHK2 mutation, or loss of expression, that occurs in PCa leads to sustained
760 AR transcriptional activity, an increase in DDR gene transcripts, and survival in response
761 to DNA damage.
762

763 **Supplemental Figure 1. AR S81 and S308 phosphorylation are not altered with**
764 **radiation.** LNCaP and C4-2 cells were seeded in serum-supplemented growth media for
765 48hrs, exposed to 6Gy IR, and AR was immunoprecipitated using a magnetic bead
766 system 1hr after radiation from 1mg cell extracts. Proteins were separated by 7.5% SDS-
767 PAGE, and blotted with AR, pAR S81, pAR S308, CHK2, and ERK1/2. Representative
768 blots are shown. Plotted is the pAR signal normalized to total AR and compared to
769 untreated cells, n=3, no statistical difference between the groups by ANOVA. Blots were
770 quantitated on Odyssey LICOR imaging system.
771
772 **Supplemental Figure 2. CHK2 Knockdown does not alter DNA breaks.** LNCaP (A)
773 and Rv1 (B) cells were seeded in whole media for 48 hours, irradiated at the indicated
774 doses at the specified times and processed for comet assays. Show is the percent DNA
775 in the comet tail, comet tail length, and tail moment (% DNA x tail length). n=3 for LNCaP
776 and n=2 for Rv1. At the same irradiation conditions there was no statistical difference
777 between vector control and CHK2 knockdown by ANOVA and Tukey's multiple
778 comparisons test.
779
780 **Supplemental Figure 3. Automated quantitation of foci.** Confocal images are captured
781 on a Zeiss LSM 880 at 40x oil using Zeiss Zen digital imaging software and processed
782 into individual TIFF files for each fluorescence channel using ImageJ. An outline of nuclei
783 (mask) from DAPI channel is created and applied to all channels. Macro-enabled image
784 processing to measure multiple fluorescence parameters with background subtraction
785 using the following parameters: area, IntDen, RawIntDen, Min and Max, fluorescence,

786 background, foci count, CorDen, CorMean. gH2AX signal is the product of foci number
787 per nuclei and mean grey value (IntDen/area).
788
789 **Supplemental Figure 4. CHK2 Knockdown does not alter hormone induced**
790 **phospho-γH2AX foci.** LNCaP and Rv1 cells were transduced with lentiviral particles
791 expressing empty vector or CHK2 shRNA on fibronectin-coated coverslips in whole media
792 for 48hrs. Media was changed to CSS overnight and cells were treated with hormone for
793 6 hours. Plotted is the number of phospho-γH2AX foci per cell. n=3 for LNCaP and n=2
794 for Rv1. At the same dose of hormone there was no statistical difference between vector
795 control and CHK2 knockdown by ANOVA and Tukey's multiple comparisons test.

A.


Targets
Flag-wtAR
Flag-ERK2
V5-wtCHK2
Flag
Flag
V5
Lysate
Flag-wtAR
Flag-ERK2
Mock
110kDa
42kDa
V5
wtAR
ERK2
Flag IP:V5-CHK2 Probe

B.

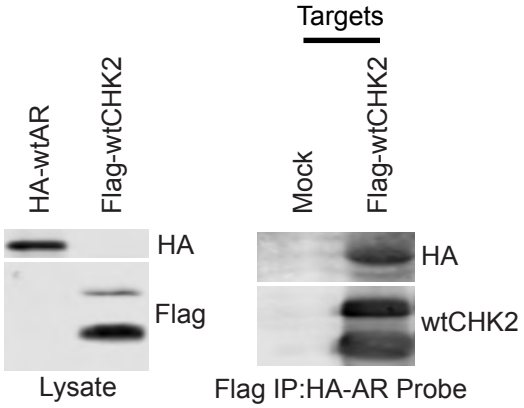

Targets
HA-wtAR
Flag-wtCHK2
HA
Flag
Lysate
Mock
Flag-wtCHK2
HA
wtCHK2
Flag IP:HA-AR Probe

Figure 1

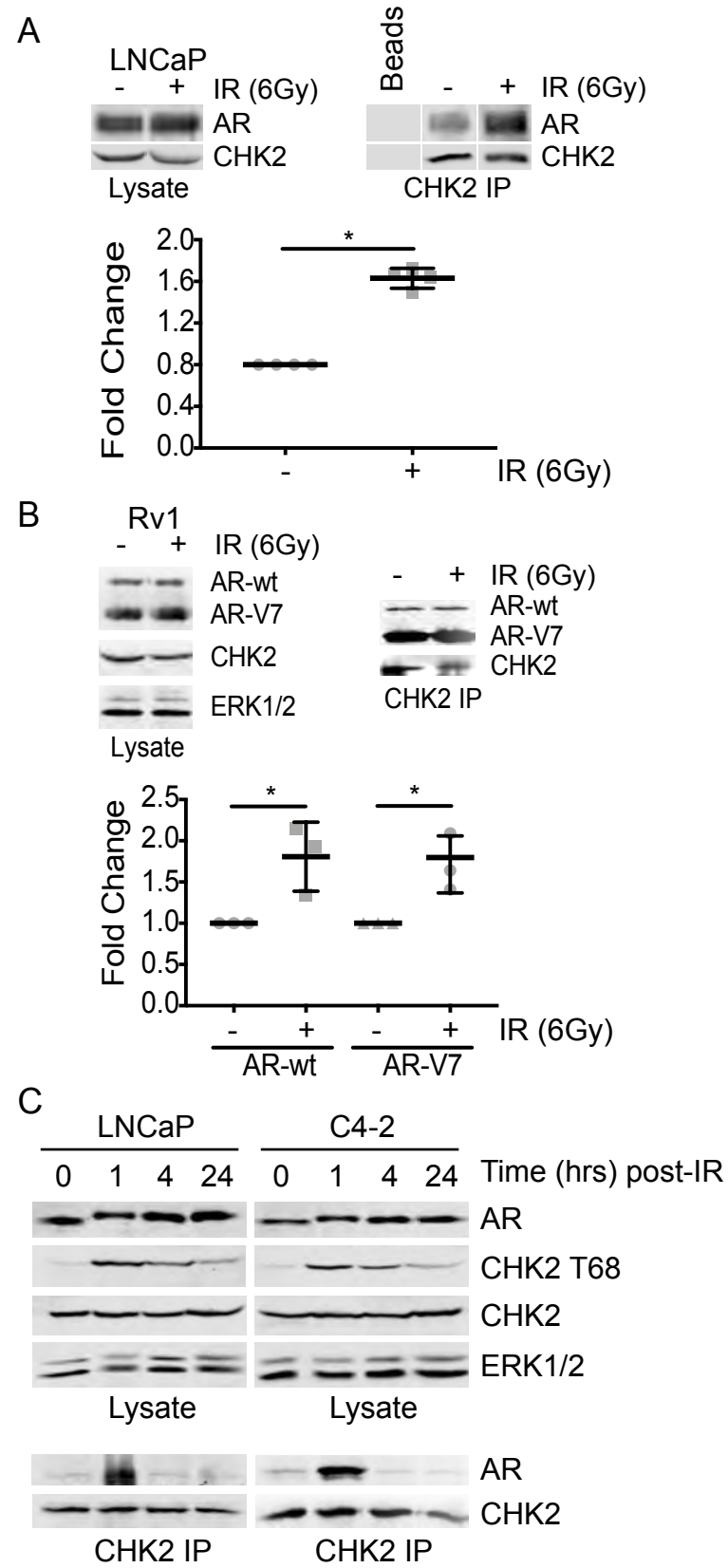
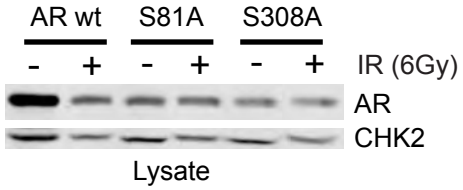
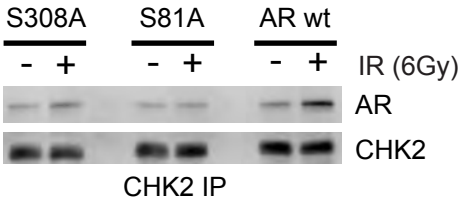
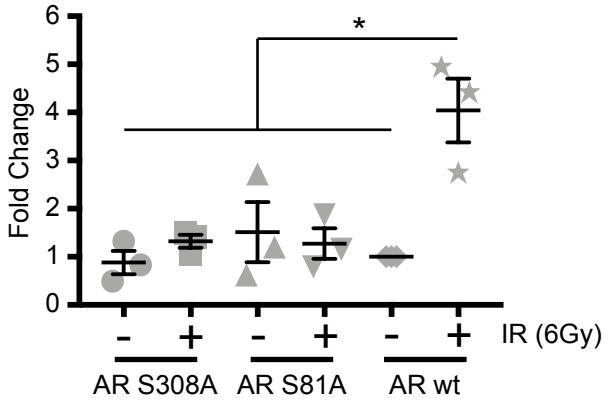

A
LNCaP
- + IR (6Gy)
AR
CHK2
Lysate
Beads
- + IR (6Gy)
AR
CHK2
CHK2 IP
Fold Change
2.0
1.6
1.2
0.8
0.4
0.0
*
- + IR (6Gy)
B
Rv1
- + IR (6Gy)
AR-wt
AR-V7
CHK2
ERK1/2
Lysate
- + IR (6Gy)
AR-wt
AR-V7
CHK2
CHK2 IP
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
*
- + - + IR (6Gy)
AR-wt AR-V7
C
LNCaP C4-2
0 1 4 24 0 1 4 24 Time (hrs) post-IR
AR
CHK2 T68
CHK2
ERK1/2
Lysate Lysate
AR
CHK2
CHK2 IP CHK2 IP
D
Fold Change
15
10
5
0
-5
*
*
*
LNCaP
0 1 4 24
Fold Change
15
10
5
0
*
*
*
C4-2
0 1 4 24

Figure 2

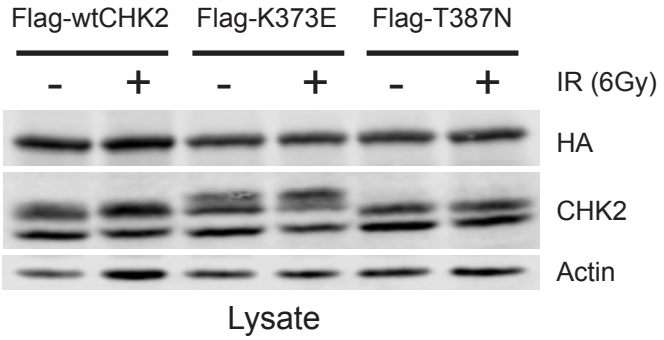
A

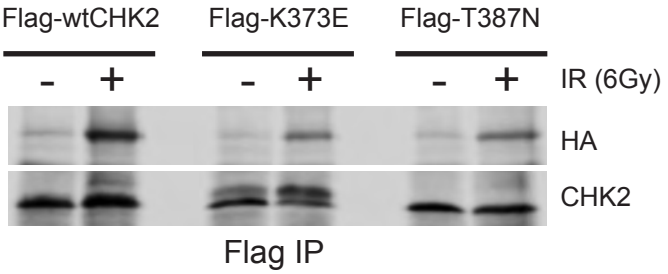

AR wt
S81A
S308A
- + - + - +
IR (6Gy)
AR
CHK2
Lysate


S308A
S81A
AR wt
- + - + - +
IR (6Gy)
AR
CHK2
CHK2 IP


Fold Change
0
1
2
3
4
5
6
*
- + - + - +
IR (6Gy)
AR S308A
AR S81A
AR wt

B


Flag-wtCHK2
Flag-K373E
Flag-T387N
- + - + - +
IR (6Gy)
HA
CHK2
Actin
Lysate


Flag-wtCHK2
Flag-K373E
Flag-T387N
- + - + - +
IR (6Gy)
HA
CHK2
Flag IP

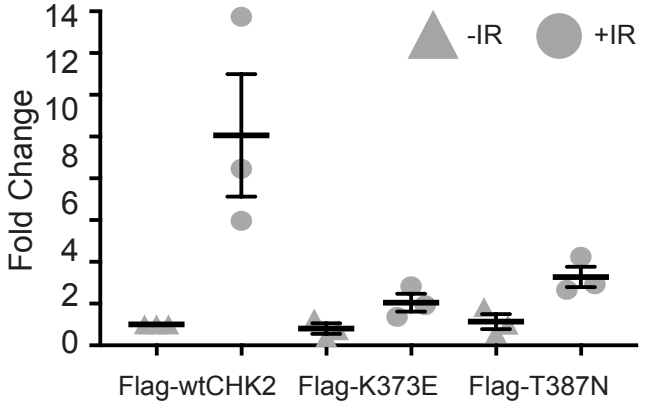
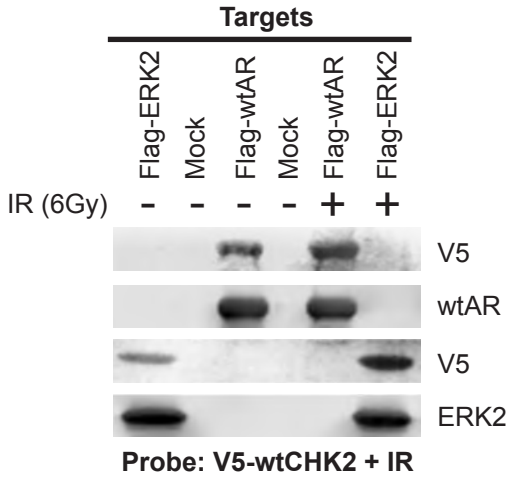
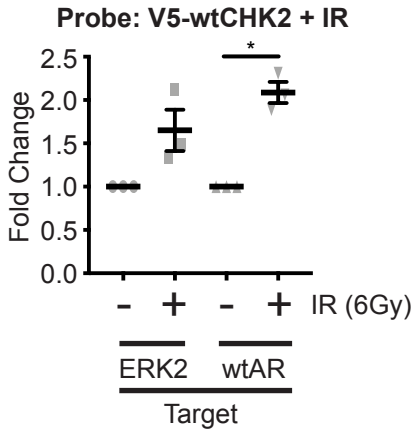

Fold Change
0
2
4
6
8
10
12
14
-IR
+IR
Flag-wtCHK2
Flag-K373E
Flag-T387N

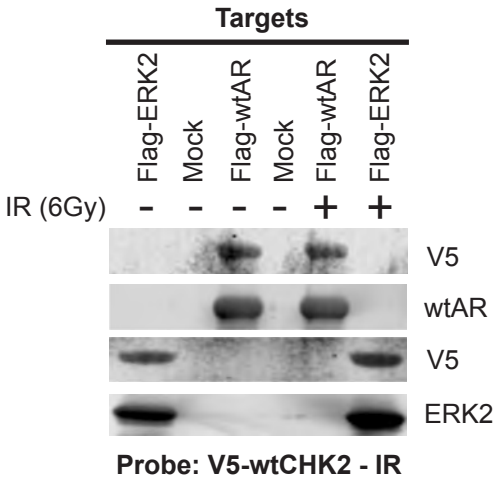
Figure 3

A


Targets
Flag-ERK2
Mock
Flag-wtAR
Mock
Flag-wtAR
Flag-ERK2
IR (6Gy)
- - - - + +
V5
wtAR
V5
ERK2
Probe: V5-wtCHK2 + IR


Probe: V5-wtCHK2 + IR
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
*
- + - + IR (6Gy)
ERK2
wtAR
Target

B


Targets
Flag-ERK2
Mock
Flag-wtAR
Mock
Flag-wtAR
Flag-ERK2
IR (6Gy)
- - - - + +
V5
wtAR
V5
ERK2
Probe: V5-wtCHK2 - IR

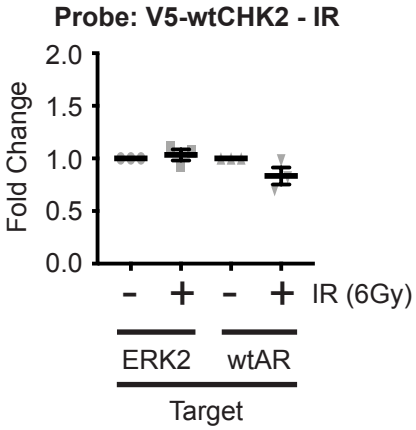
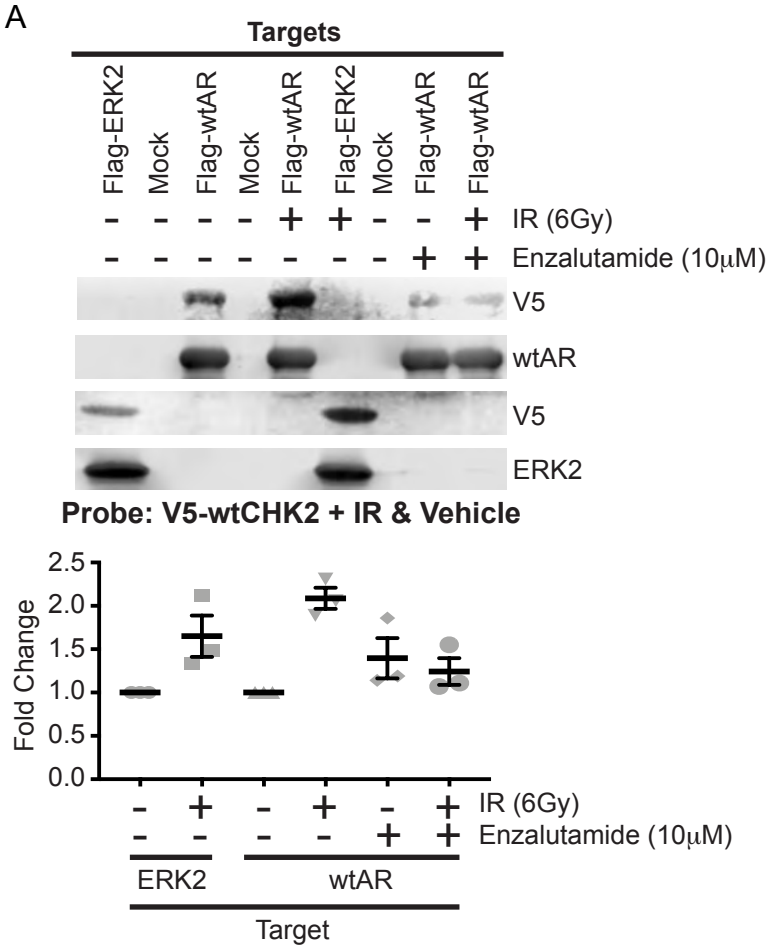

Probe: V5-wtCHK2 - IR
Fold Change
2.0
1.5
1.0
0.5
0.0
- + - + IR (6Gy)
ERK2
wtAR
Target

Figure 4

A


Targets
Flag-ERK2
Mock
Flag-wtAR
Mock
Flag-wtAR
Flag-ERK2
Mock
Flag-wtAR
Flag-wtAR
IR (6Gy)
Enzalutamide (10μM)
V5
wtAR
V5
ERK2
Probe: V5-wtCHK2 + IR & Vehicle
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
IR (6Gy)
Enzalutamide (10μM)
ERK2
wtAR
Target

B

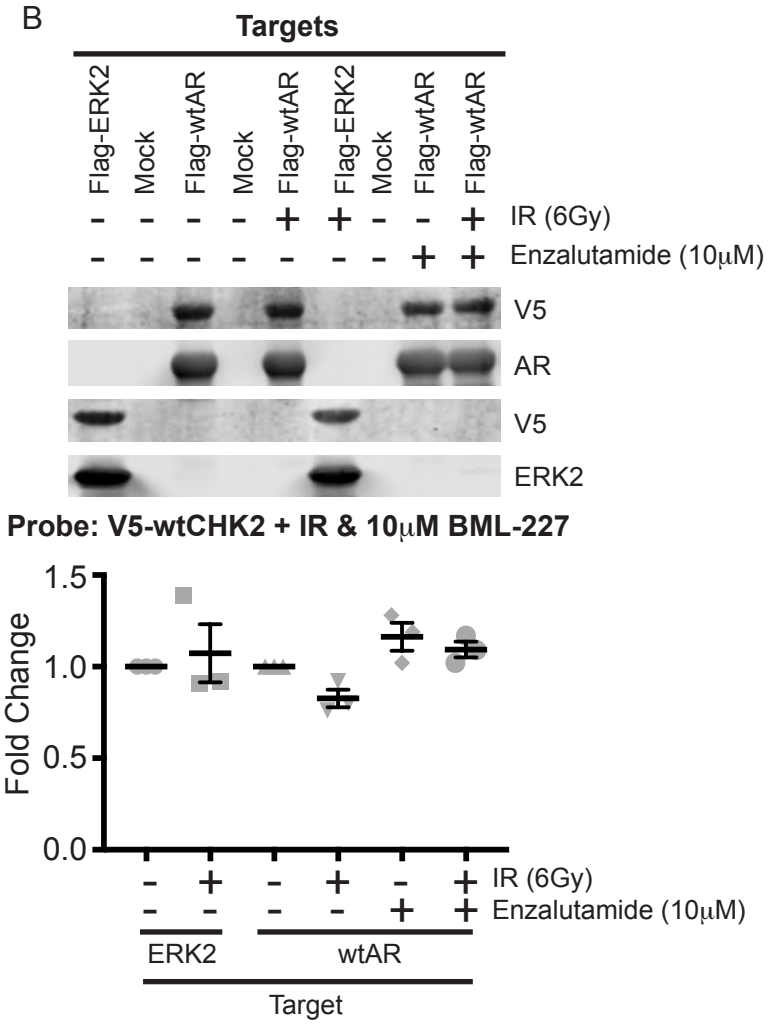
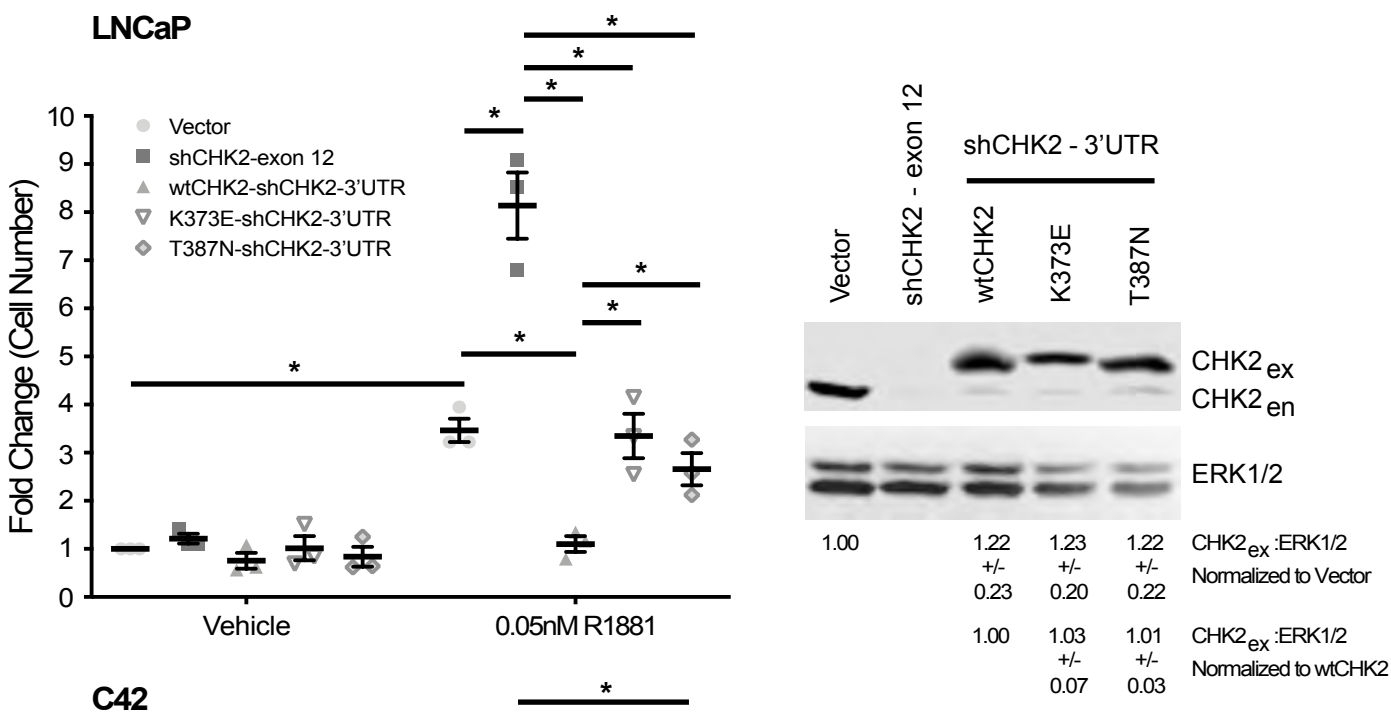
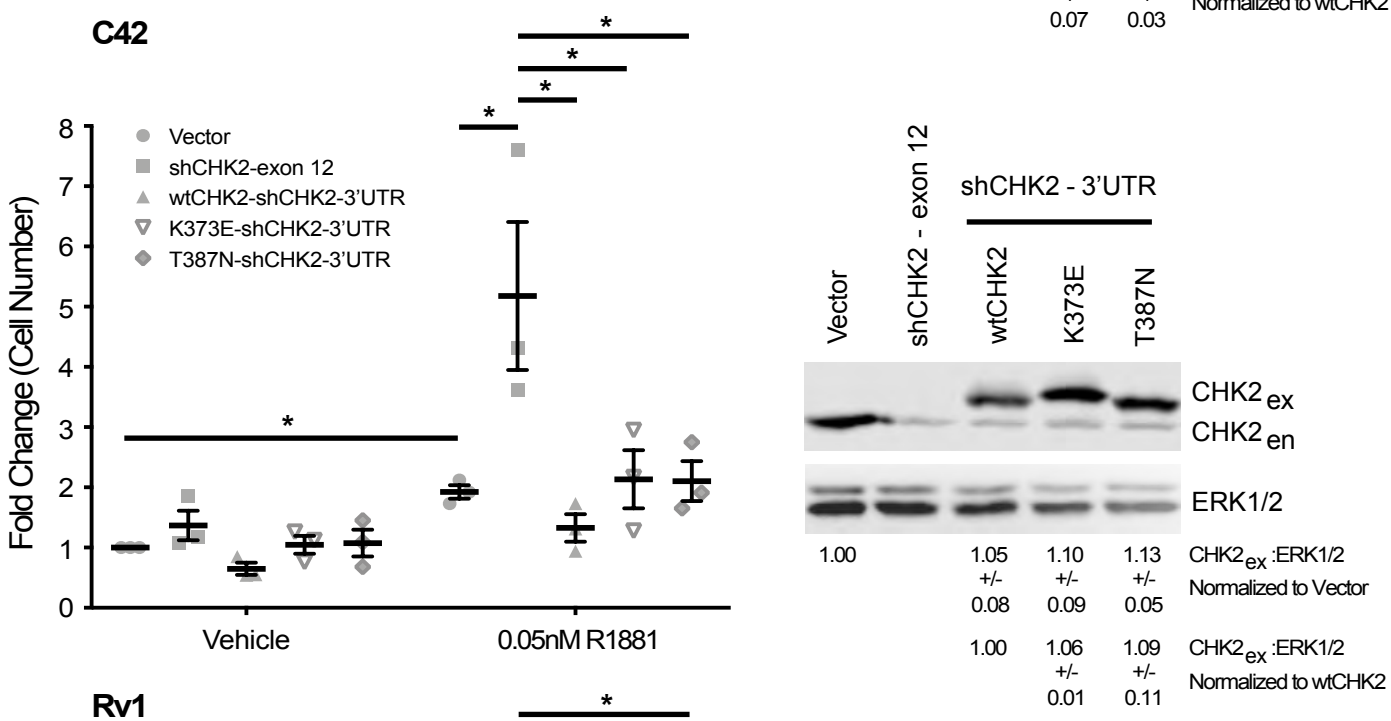

Targets
Flag-ERK2
Mock
Flag-wtAR
Mock
Flag-wtAR
Flag-ERK2
Mock
Flag-wtAR
Flag-wtAR
IR (6Gy)
Enzalutamide (10μM)
V5
AR
V5
ERK2
Probe: V5-wtCHK2 + IR & 10μM BML-227
Fold Change
1.5
1.0
0.5
0.0
IR (6Gy)
Enzalutamide (10μM)
ERK2
wtAR
Target

Figure 5

A.


LNCaP
Vector
shCHK2-exon 12
wtCHK2-shCHK2-3'UTR
K373E-shCHK2-3'UTR
T387N-shCHK2-3'UTR
Fold Change (Cell Number)
Vehicle
0.05nM R1881
shCHK2 - 3'UTR
Vector
shCHK2 - exon 12
wtCHK2
K373E
T387N
CHK2 ex
CHK2 en
ERK1/2
1.00
1.22 +/- 0.23
1.23 +/- 0.20
1.22 +/- 0.22
CHK2ex :ERK1/2 Normalized to Vector
1.00
1.03 +/- 0.07
1.01 +/- 0.03
CHK2ex :ERK1/2 Normalized to wtCHK2

B.


C42
Vector
shCHK2-exon 12
wtCHK2-shCHK2-3'UTR
K373E-shCHK2-3'UTR
T387N-shCHK2-3'UTR
Fold Change (Cell Number)
Vehicle
0.05nM R1881
shCHK2 - 3'UTR
Vector
shCHK2 - exon 12
wtCHK2
K373E
T387N
CHK2 ex
CHK2 en
ERK1/2
1.00
1.05 +/- 0.08
1.10 +/- 0.09
1.13 +/- 0.05
CHK2ex :ERK1/2 Normalized to Vector
1.00
1.06 +/- 0.01
1.09 +/- 0.11
CHK2ex :ERK1/2 Normalized to wtCHK2

C.

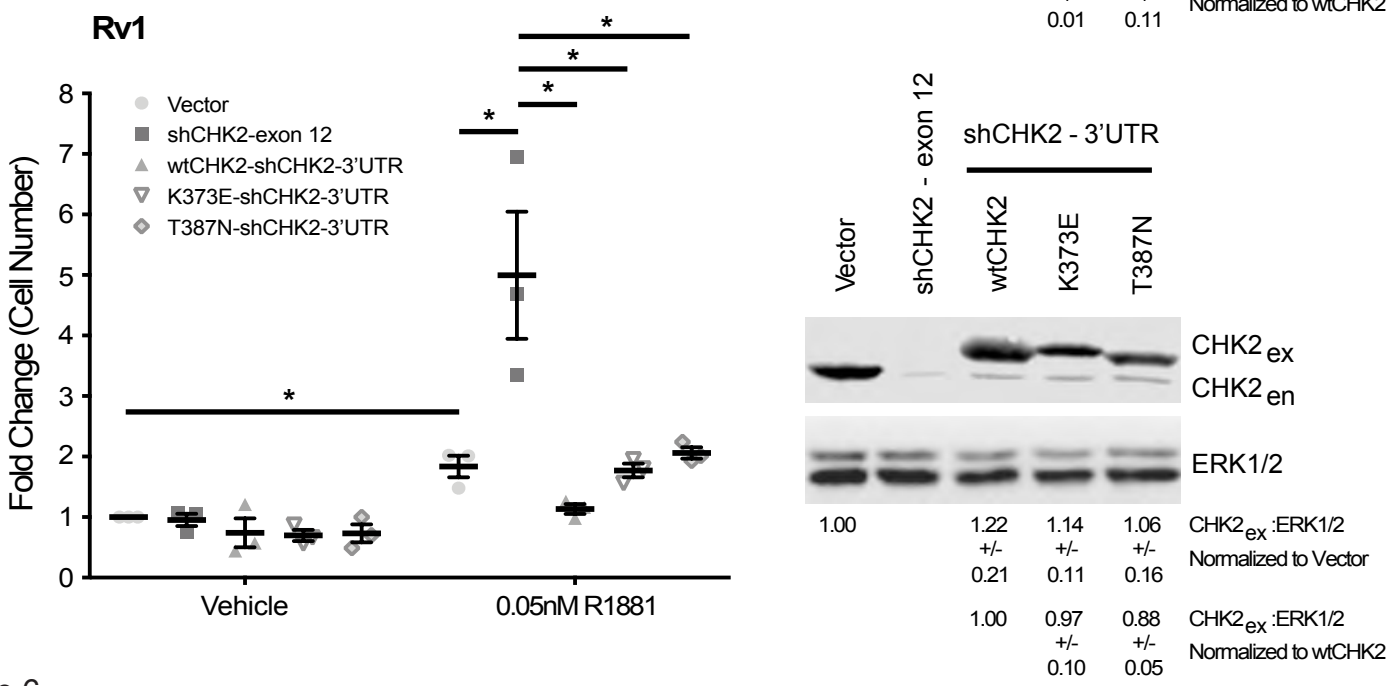
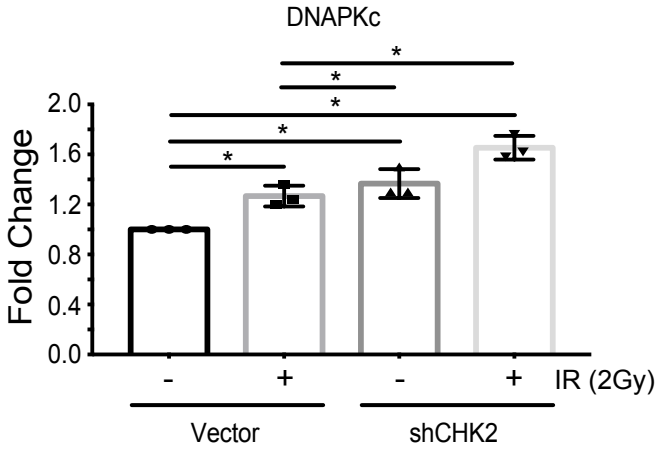

Rv1
Vector
shCHK2-exon 12
wtCHK2-shCHK2-3'UTR
K373E-shCHK2-3'UTR
T387N-shCHK2-3'UTR
Fold Change (Cell Number)
Vehicle
0.05nM R1881
shCHK2 - 3'UTR
Vector
shCHK2 - exon 12
wtCHK2
K373E
T387N
CHK2 ex
CHK2 en
ERK1/2
1.00
1.22 +/- 0.21
1.14 +/- 0.11
1.06 +/- 0.16
CHK2ex :ERK1/2 Normalized to Vector
1.00
0.97 +/- 0.10
0.88 +/- 0.05
CHK2ex :ERK1/2 Normalized to wtCHK2

Figure 6

A.


DNAPKc
Fold Change
2.0
1.6
1.2
0.8
0.4
0.0
*
*
*
*
*
-
+
-
+
IR (2Gy)
Vector
shCHK2

B.

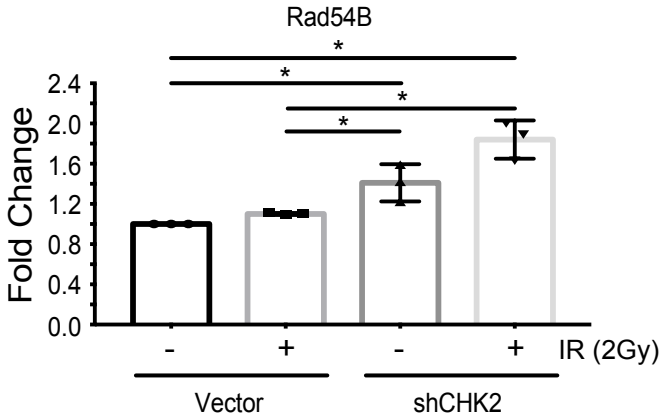
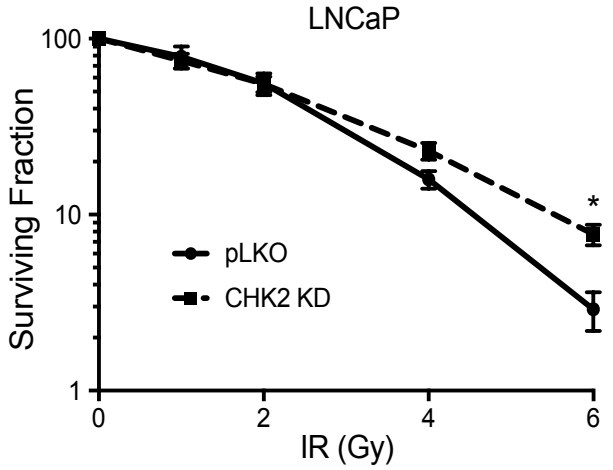
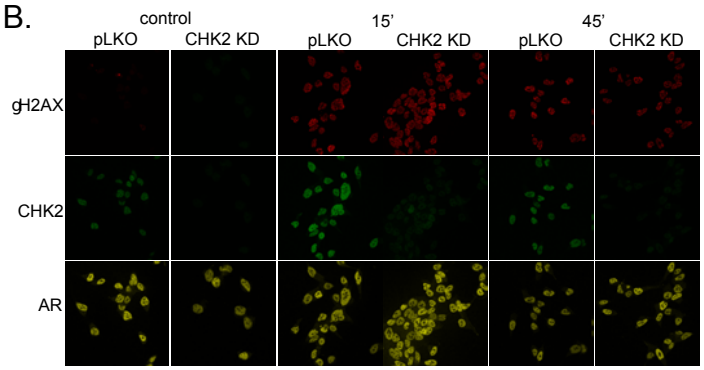

Rad54B
Fold Change
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
*
*
*
-
+
-
+
IR (2Gy)
Vector
shCHK2

Figure 7

A.


LNCaP
Surviving Fraction
100
10
1
0
2
4
6
IR (Gy)
pLKO
CHK2 KD
*

B.


control
15'
45'
pLKO
CHK2 KD
pLKO
CHK2 KD
pLKO
CHK2 KD
gH2AX
CHK2
AR

C.

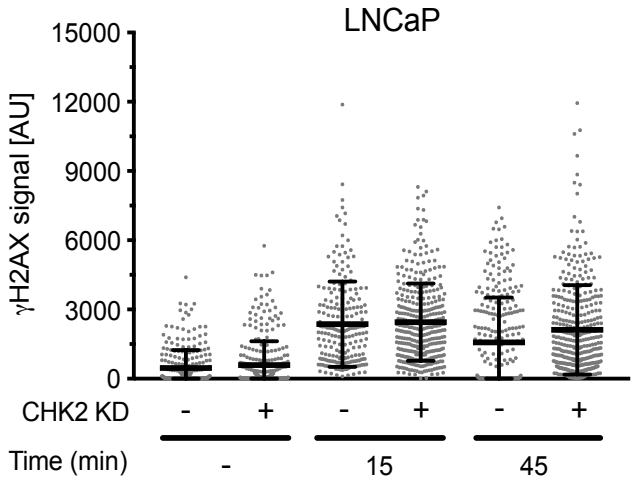

LNCaP
γH2AX signal [AU]
15000
12000
9000
6000
3000
0
CHK2 KD
-
+
-
+
-
+
Time (min)
-
15
45

Figure 8

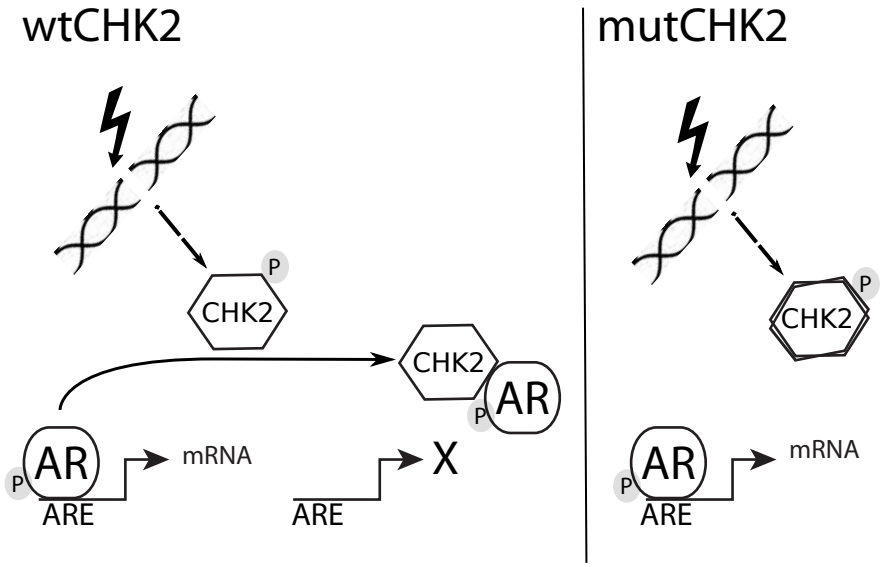
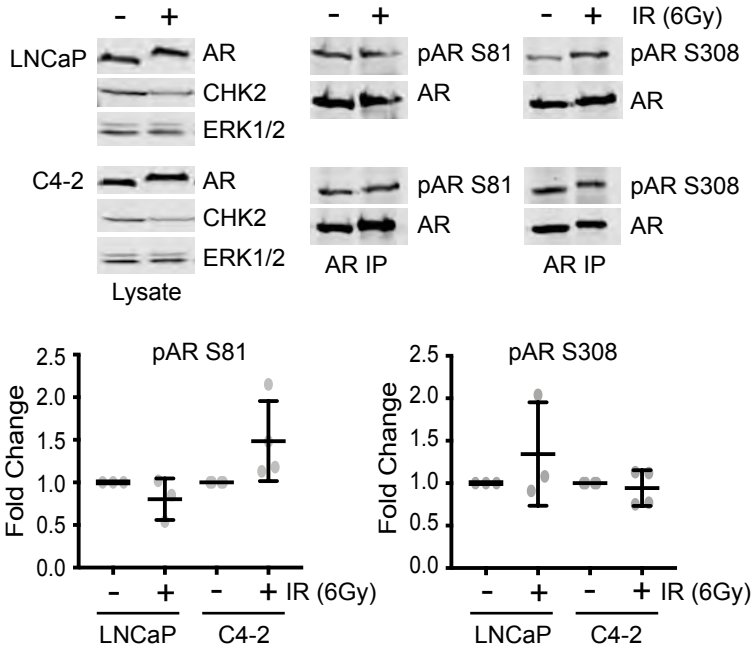
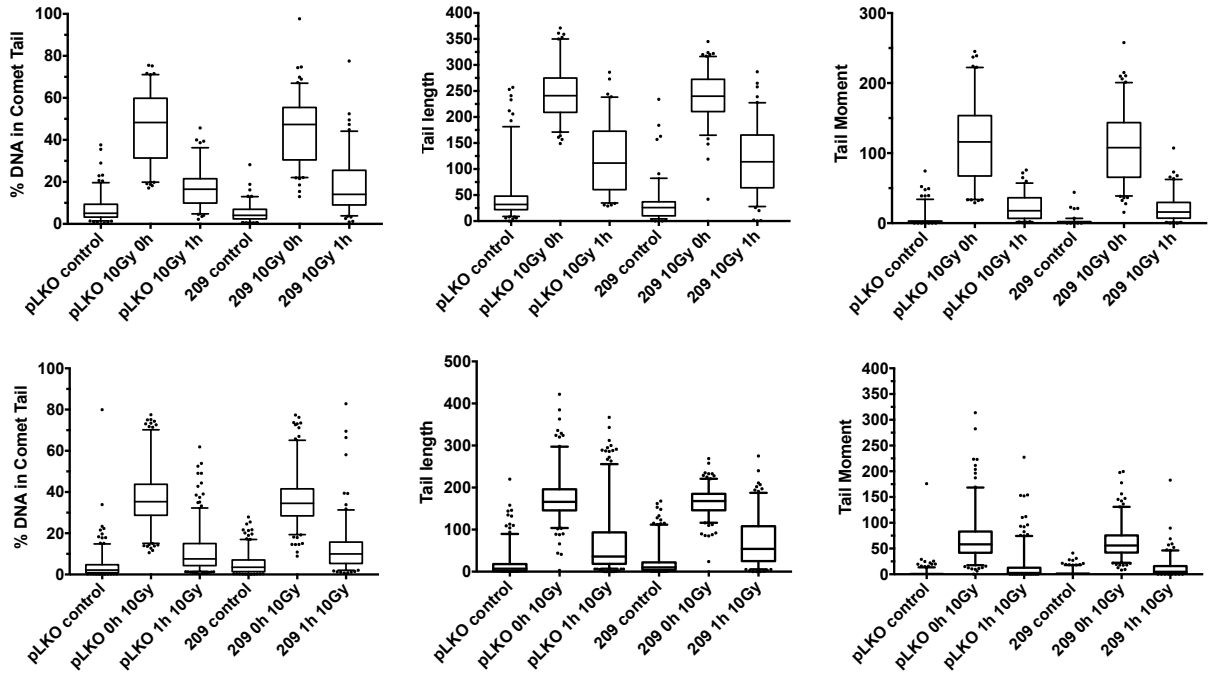

wtCHK2
mutCHK2
P
CHK2
CHK2
AR
P
AR
P
mRNA
ARE
X
ARE
P
CHK2
AR
P
mRNA
ARE

Figure 9

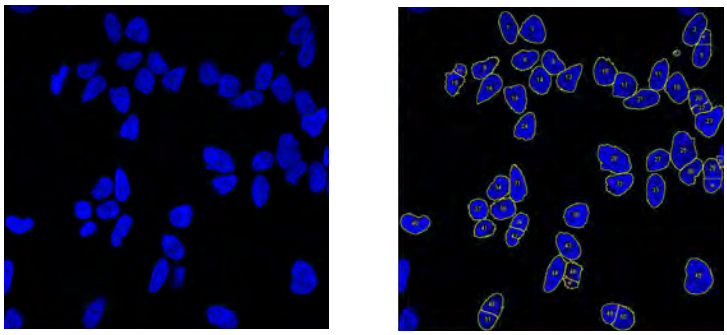


Supplemental Figure 1

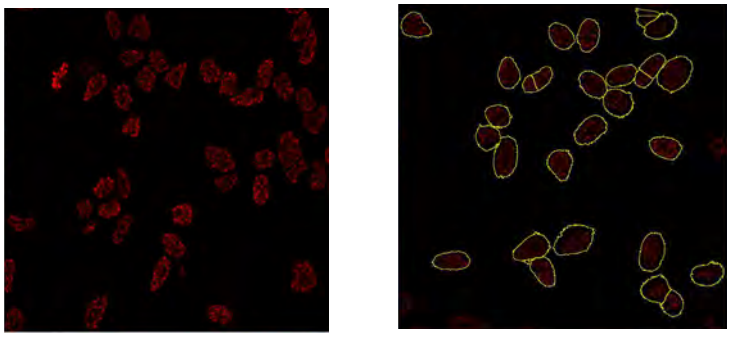


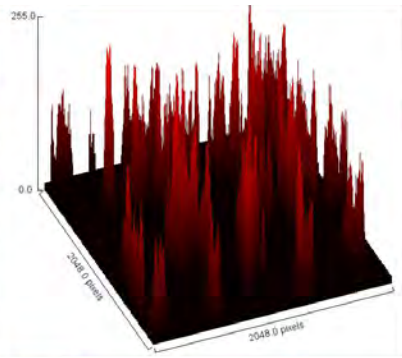
Supplemental Figure 2

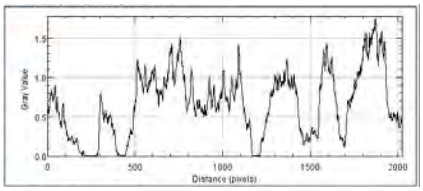
1) Create a mask from DAPI channel



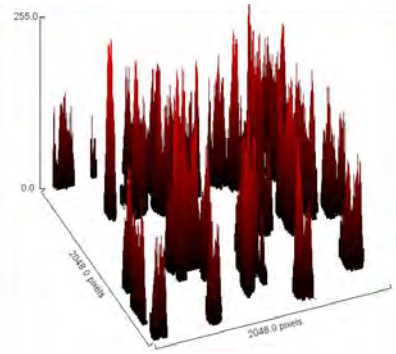
2) Apply DAPI mask onto the gH2AX channel

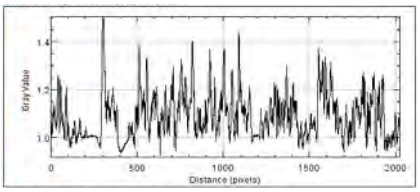




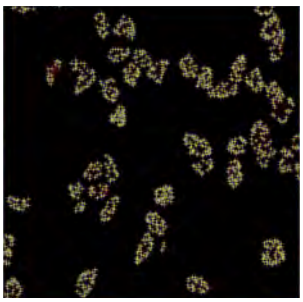


3) Background subtraction



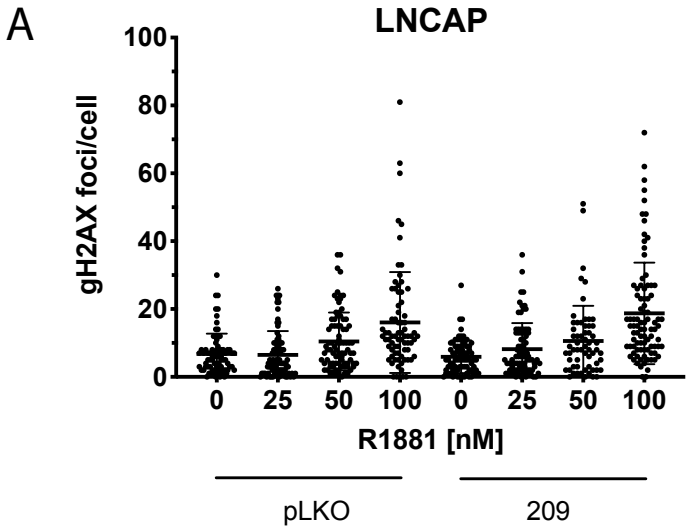


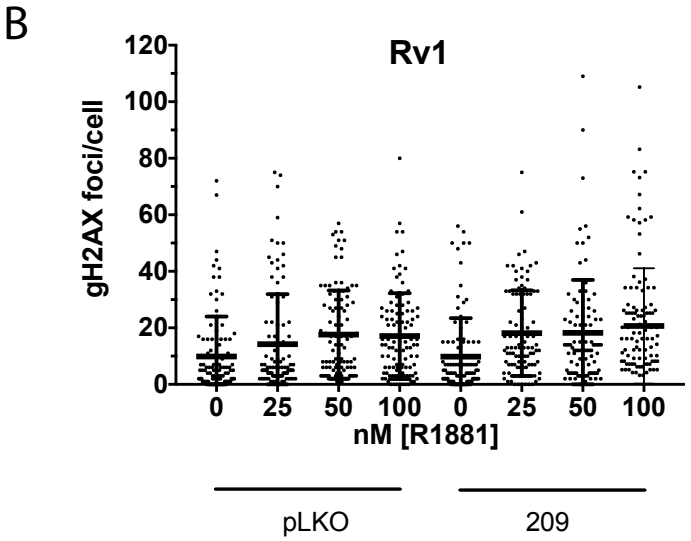
4) Maxima points (foci)



Example of output

Supplemental Figure 3





Supplemental Figure 4