

1 ***ESR1* mutant breast cancers show elevated basal cytokeratins and immune**  
2 **activation**

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28 **Abstract**

29 Estrogen receptor alpha (ER/*ESR1*) is mutated in 30-40% of endocrine resistant ER-  
30 positive (ER+) breast cancer. *ESR1* mutations cause ligand-independent growth and  
31 increased metastasis *in vivo* and *in vitro*. Despite the distinct clinical features and  
32 changes in therapeutic response associated with *ESR1* mutations, there are no data  
33 about their potential role in intrinsic subtype switching. Applying four luminal and  
34 basal gene set pairs, *ESR1* mutant cell models and clinical samples showed a  
35 significant enrichment of basal subtype markers. Among them, the six basal  
36 cytokeratins (BCKs) were the most enriched genes. Induction of BCKs was  
37 independent of ER binding and instead associated with chromatin reprogramming  
38 centered around a progesterone receptor-orchestrated topological associated  
39 domain at the *KRT14/16/17* genomic region. Unexpectedly, high *BCK* expression in  
40 ER+ primary breast cancer is associated with good prognosis, and these tumors  
41 show enriched activation of a number of immune pathways, a distinctive feature  
42 shared with *ESR1* mutant tumors. S100A8 and S100A9 were among the most highly  
43 induced immune mediators shared between high-*BCKs* ER+ and *ESR1* mutant  
44 tumors, and single-cell RNA-seq analysis inferred their involvement in paracrine  
45 crosstalk between epithelial and stromal cells. Collectively, these observations  
46 demonstrate that *ESR1* mutant tumors gain basal features with induction of basal  
47 cytokeratins via epigenetic mechanisms in rare subpopulation of cells. This is  
48 associated with increased immune activation, encouraging additional studies of  
49 immune therapeutic vulnerabilities in *ESR1* mutant tumors.

## 50 **Introduction**

51 Breast cancer is characterized by a high degree of heterogeneity, originally  
52 identified through the use of immunohistochemistry and gene expression profiling<sup>1,2</sup>.  
53 Broadly, molecular subtypes can be grouped into luminal (luminal A and luminal B),  
54 HER2-enriched and basal-like tumors, primarily driven by expression of ER, PR and  
55 HER2 and Ki67<sup>3</sup>. Tumors with different molecular subtypes show distinguishing  
56 clinical features and therapeutic responses<sup>4,5</sup>, including metastatic spread and  
57 immune profiles<sup>6,7</sup>.

58 The basal-like subtype, which represents 15-25% of all cases and overlaps  
59 with triple negative breast cancers (TNBC), is characterized by a unique gene  
60 expression profile similar to that of myoepithelial normal mammary cells<sup>8</sup>. Basal-like  
61 breast cancers are more aggressive and patients suffer from shorter metastases-  
62 free survival compared to those with luminal subtypes<sup>8,9</sup>. Mechanisms underlying  
63 increased invasive properties of basal-like tumors include deregulation of the  
64 CCL5/CCR5 axis<sup>10</sup>, amplified EGFR<sup>11</sup> kinase signaling and activation of TGF- $\beta$   
65 signaling<sup>12</sup>. Despite multiple signaling aberrations providing challenges for efficient  
66 therapeutic strategies, recent studies have unveiled unique vulnerabilities of basal-  
67 like breast cancers, such as higher levels of PD-L1 expression along with  
68 constitutive IFN $\gamma$  signaling activation<sup>13</sup>, in line with higher immune- infiltration  
69 scores<sup>6</sup>. While the FDA has granted an accelerated approval for atezolizumab, a  
70 monoclonal antibody drug targeting PD-L1, plus chemotherapy for the treatment of  
71 TNBC<sup>14</sup>, the potential application of immune therapies for patients with luminal  
72 breast cancer remains largely unknown.

73           Among the four intrinsic subtypes, basal and luminal subtypes show opposite  
74   histochemical features and notable differences in prognosis<sup>15,16</sup>, however there is  
75   increasing evidence that these subtypes are on a continuum of “luminal-ness” and  
76   “basal-ness” features. Models of breast cancer lineage evolution describe that basal  
77   and luminal progenitor cells are derived from the same bipotential progenitors<sup>17</sup>,  
78   indicating the potential of lineage reprogramming during cancer progression. Such  
79   subtype switching during tumor evolution has been described and is critical for  
80   implementation of precision therapeutics<sup>18-20</sup>. A recent study by Bi et al. reported  
81   loss of luminal and gain of basal markers in endocrine resistant breast tumors<sup>21</sup>.  
82   Mechanisms underlying the intrinsic subtype plasticity are largely unknown, with  
83   some exceptions. *JARID1B*<sup>22</sup> and *ARID1A*<sup>23</sup> have been described as essential  
84   luminal lineage driver genes and their mutations result in luminal-to-basal subtypes  
85   switches. In addition, enhancer reprogramming at GATA3 and AP1 binding sites has  
86   been highlighted as a pivotal epigenetic mechanism allowing lineage plasticity<sup>21</sup>.

87           ER is well characterized as a luminal lineage marker<sup>24</sup>. Hotspot mutations in  
88   its ligand-binding domain occur in 30%-40% of endocrine resistant breast tumors,  
89   promoting ligand-independent ER activation and metastasis<sup>25-27</sup>. Several recent  
90   studies showed that *ESR1* mutant tumors are not only associated with endocrine  
91   resistance, but also gain unexpected resistance towards CDK4/6 inhibitors<sup>28</sup>, mTOR  
92   inhibitors<sup>29</sup> and radiation therapy<sup>30</sup> in a mutation subtype and context dependent  
93   manner, suggesting potentially more complex re-wiring of ER mutant tumors.

94           We set out to examine whether *ESR1* mutations alter the “luminal-ness” and  
95   “basal-ness” balance in breast cancer cell line models and clinical specimens. We

96 discovered that ER mutant tumors gain basal-like features, characterized by  
97 elevated expression of basal cytokeratins as a result of epigenetic reprogramming.  
98 Immune context analyses in clinical specimens revealed potential therapeutic  
99 vulnerabilities accompanying the increased basal-ness in *ESR1* mutant breast  
100 cancer, a finding of potential clinical relevance.

## 101 **Results**

### 102 **Basal gene signatures are enriched in *ESR1* mutant breast cancer**

103 To examine whether *ESR1* mutations alter “luminal-ness” and “basal-ness”  
104 we utilized four independent luminal and basal gene signatures (Fig. 1A,  
105 Supplementary Table S1). Gene sets from Charafe-Jauffret et al.<sup>31</sup> and Huper et  
106 al.<sup>32</sup> were obtained from MSigDB (Supplementary Fig. S1A and S1B), and in  
107 addition we generated two other gene sets from i) intrinsic subtype genes<sup>33</sup>  
108 differentially expressed between luminal (n=33) and basal (n=39) breast cancer cell  
109 lines (Supplementary Table S2)<sup>34-36</sup> and ii) genes differentially expressed between  
110 luminal and basal primary tumors in TCGA<sup>37</sup> (Supplementary Fig. S1C and S1D).  
111 Although the overlap among the different gene sets was limited (Fig.1B), likely  
112 reflecting differences in methodology and sources, some well described lineage  
113 marker genes (e.g. *ESR1* and *FOXA1* as luminal markers, and *KRT6A* and *KRT16*  
114 as basal markers) were observed in 3 out of 4 gene sets.

115 As expected, all four basal gene sets were significantly enriched in basal  
116 versus luminal breast cancer cell lines and tumors (Supplementary Fig. S2A and  
117 S2B), and *vice versa* for luminal gene sets except for the Huper luminal markers,  
118 likely due to its derivation from normal mammary tissue (Supplementary Fig. S2C  
119 and S2D). We found concordantly increased enrichment of basal gene sets in  
120 Y537S and D538G MCF7 *ESR1* genome-edited mutant cells, whereas no  
121 differences were observed in estrogen treated *ESR1* wildtype cells (Fig. 1C). In  
122 contrast, we did not observe a consistent change in the luminal gene sets (Fig. 1D).  
123 The enrichment of the basal gene sets in the *ESR1* mutant cells was also seen in an

124 independent CRISPR-engineered MCF7 *ESR1* mutant cell model recently reported  
125 by Arnesen et al<sup>38</sup> (Supplementary Fig. S3A) and in our T47D *ESR1* mutant cells<sup>27</sup>  
126 (Supplementary Fig. S3B). Of note, no consistent and strong alterations of luminal  
127 and basal gene sets enrichment levels were detected in *ESR1* WT endocrine  
128 resistant ER+ breast cancer cell models<sup>21,39-46</sup> (8 tamoxifen resistant, 2 fulvestrant  
129 resistant and 7 long-term estradiol deprivation (LTED) models), suggesting that the  
130 “basal-ness” shift is a unique feature acquired as a result of *ESR1* mutations  
131 (Supplementary Fig. S3C)<sup>46</sup>.

132 We next sought to extend our findings to clinical specimens using RNA-seq  
133 data composed of 51 intra-patient matched ER+ primary-metastatic tumor pairs (7  
134 *ESR1* mutant and 44 *ESR1* WT pairs) (Supplementary Table S3). Similar to  
135 observations in cell lines, *ESR1* mutant metastatic breast cancers showed a  
136 significant enrichment of basal gene signatures compared to tumors with WT *ESR1*  
137 (Fig. 1E). We did not observe a concurrent decrease of luminal markers (Figure 1F).  
138 Taken together, these findings suggested a novel and unexpected gain of “basal-  
139 ness” in *ESR1* mutant tumors.

140

## 141 **Basal cytokeratins are elevated in *ESR1* mutant breast cancer cells and** 142 **tumors**

143 We next interrogated the union of the four basal gene sets (N=634) to identify  
144 which basal marker genes were consistently induced in *ESR1* mutant breast cancer  
145 cells. Integrating RNA-seq results from MCF7 cell models<sup>27</sup> and clinical samples  
146 identified a group of basal cytokeratins (*KRT5*, *KRT6A*, *KRT6B*, *KRT14*, *KRT16*, and

147 *KRT17*) as the top consistently increased basal markers (Fig. 2A, Supplementary  
148 Fig. S4A and Supplementary Table S4). Elevated basal cytokeratins (BCKs) mRNA  
149 levels were further confirmed in independent qRT-PCR experiment in *ESR1* mutant  
150 MCF7 cells (Fig. 2B). Analyzing fold-change expression of all basal markers in a  
151 number of MCF7 *ESR1* mutant cell models previously described<sup>25,27,38</sup> revealed  
152 *KRT5, 16* and *17* as the top increased basal genes (Supplementary Fig. S4B-D). In  
153 the T47D *ESR1* mutant cells, *KRT16* was significantly increased (Supplementary  
154 Fig. S4E), but the observed enrichment of basal marker genes (Supplementary Fig.  
155 S3B) was also driven by other non-canonical basal genes such as *WLS* and *HTRA1*  
156 (Supplementary Table S5), suggesting some context-dependent mechanisms for the  
157 increased basal-ness.

158 We also queried *KRT* expression in overexpression models. In MCF7 cells  
159 with stable overexpression of HA-tagged WT and mutant ER (Y537S and D538G)  
160 (Supplementary Figure S5A and S5B), we again observed significant  
161 overexpression of *KRT5, KRT6A, KRT6B, KRT16*, and *KRT17* (Supplementary Fig.  
162 S5C).

163 Given higher BCK mRNA expression in *ESR1* mutant cells, we examined  
164 their expression at the protein level. We confirmed higher CK5 and CK16 protein  
165 levels in early passage (P6-8) *ESR1* mutant cells, but curiously expression was not  
166 detectable in later passages (P30-32) (Supplementary Fig. S6A). This finding was  
167 consistent with prior reports on slower growth of CK5+ sub-populations<sup>47</sup>, reflecting  
168 selection forces eliminating BCK-positive subclones from luminal cell populations. To  
169 determine whether BCK expression was limited to minor sub-populations in *ESR1*



170 mutant cells, we performed IF staining for CK5, CK16 and CK17 in early passage  
171 cells (below P12) (Fig. 2C). No BCK positive clones were identified in MCF7-WT  
172 cells, while 0.5-1% of Y537S and D538G *ESR1* mutant cells exhibited strong diffuse  
173 cytoplasmic CK5/16/17 expression. In addition, 3-5% of *ESR1* mutant cells  
174 displayed strong BCK signals localized as foci adjacent to the nucleus  
175 (Supplementary Fig. S6B), and this was again not observed in the WT cells.  
176 Furthermore, co-staining of CK5+CK16 and CK16+CK17 showed that the BCK  
177 proteins were predominantly (in 75%-90% imaged cells) upregulated in the same  
178 sub-population of cells (Supplementary Fig. S6C and S6D). In contrast, luminal  
179 cytokeratin CK8 was homogenously expressed with stronger expression at the  
180 edges of each cell cluster (Supplementary Fig. S6E), suggesting that the marked  
181 heterogeneity was a unique feature for BCK expression in the luminal cell  
182 background.

183

#### 184 **BCK induction is independent of mutant ER DNA binding but requires low ER** 185 **expression**

186 Mutant ER can function in a ligand-independent manner<sup>26,27</sup>, and we thus  
187 tested whether induction of BCKs resulted from ligand-independent ER activity. We  
188 interrogated eight publicly available RNA-seq and microarray data sets with estradiol  
189 (E2) treatment in six different ER+ breast cancer cell lines<sup>26,27,48-51</sup>. In contrast to  
190 strong E2 induction of classical ER target genes such as *GREB1*, *TFF1* and *PGR*,  
191 expression of basal and luminal cytokeratins genes was not regulated by E2 with the  
192 exception of *KRT7* (Fig. 3A). We then examined whether BCK expression was

193 regulated via *de novo* genomic binding of mutant ER at BCK genes. We performed  
194 ChIP-seq in MCF7 WT and *ESR1* mutant cells in the absence and presence of E2.  
195 As expected, in the absence of E2 we detected very few ER binding sites in WT  
196 MCF7 cells (n=125), whereas E2 stimulation triggered substantial ER binding events  
197 (n=12,472) (Supplementary Table S6). Consistent with previous studies<sup>25,26</sup>, Y537S  
198 and D538G ER show strong ligand-independent binding, with 657 binding sites in  
199 Y537S and 1,016 in D538G mutant cells (Supplementary Fig. S7A). The *GREB1*  
200 gene locus is shown as a representative example (Fig 3B, left panel). Co-  
201 occupancy analyses between WT-E2 and mutant-vehicle sets demonstrated that  
202 one third of all Y537S (36%) and D538G (31%) ER binding sites were not detected  
203 in the WT+E2 data suggesting gain-of-function novel binding sites (Supplementary  
204 Fig. S7B); however, none of them mapped to the BCKs genes with increased  
205 expression in *ESR1* mutant cells (-/+ 50kb of transcriptional start sites) (Fig. 3B,  
206 middle and right panel).

207 We then expanded our analyses and examined potential estrogen-regulation  
208 of all basal marker genes, again using the union of the four basal gene sets (N=634).  
209 Comparison of E2 and *ESR1* mutation-conferred fold changes of these genes in  
210 MCF7 cells revealed that the top upregulated basal markers in *ESR1* mutant cells  
211 were not E2-induced (Supplementary Fig. S7C and S7D). In addition, only 20 basal  
212 genes (3%) harbor mutant ER binding sites at -/+ 50 kb of TSS (Supplementary Fig.  
213 S7E), and 18 of those were not differentially expressed between WT and mutant  
214 cells (Supplementary Fig. S7F). Taken together, these analyses suggest that the

215 shift to “basal-ness” in *ESR1* mutant cells was not mediated via ligand-independent  
216 binding of mutant ER to BCK gene loci.

217 To further understand interplay between *ESR1* and *KRT* gene expression, we  
218 determined expression of basal and luminal *KRT* genes in ER+ primary breast  
219 tumors. As shown in Figure 3C, the six BCKs were significantly negatively correlated  
220 with *ESR1* expression, whereas the luminal *KRT* were mostly positively correlated  
221 with *ESR1* (Fig. 3C). Luminal *KRT7* was again the exception, being negatively  
222 correlated with *ESR1* expression, in line with it being repressed by ER (Figure 3A).  
223 The inverse correlation between BCK and *ESR1* expression was also reflected in  
224 results from ER knockdown experiments, in which loss of *ESR1* significantly  
225 increased expression of BCKs in MCF7 WT and mutant cells (Fig. 3D). Similar  
226 results were obtained in five additional ER+ breast cancer cell lines where we  
227 observed a general increase of BCK expression after *ESR1* knockdown  
228 (Supplementary Fig. S8). In addition, co-staining of ER and CK5/CK16/17 in MCF7  
229 *ESR1* mutant cells showed significantly lower ER expression in BCK+ cells than in  
230 the surrounding BCK- cells (Fig. 3E). Collectively, these data demonstrate that ER  
231 serves as a negative regulator of BCKs expression independent of ligand and  
232 mutational status, and suggest that low ER expression is likely necessary but not  
233 sufficient to facilitate BCKs overexpression in a subpopulation of *ESR1* mutant cells.  
234 These data also support a role for mutant ER in regulating BCK expression via  
235 epigenetic regulation, a mechanism that we have recently shown to be used by  
236 mutant ER<sup>38</sup>.

237

238 **PR regulation of BCK expression through binding at a CTCF-driven chromatin**  
239 **loop at the *KRT14/16/17* loci in *ESR1* mutant cells**

240 To investigate potential epigenetic regulation of *KRT5/6A/6B* and  
241 *KRT14/16/17*, we first compared their regional epigenetic landscapes on  
242 chromosome 12 and 17, respectively, in luminal and basal breast cancer cell lines  
243 and tumors (Supplementary Fig. S9). Integrative analysis of ATAC-seq and ChIP-  
244 seq profiles of H3K4me2, H3K4me3, H3K9ac and H3K27ac suggested that these  
245 two regions are epigenetically silent in MCF7 (Supplementary Fig. S9A), consistent  
246 with low expression. In basal breast cancer cell lines and tumors, there is an  
247 enrichment of H3K27 acetylation (Supplementary Fig. S9B) and number of ATAC-  
248 seq peaks (Supplementary Fig. S9C) at BCK loci, consistent with increased mRNA  
249 expression (Supplementary Fig. S9E and S9F). This is also observed in *ESR1*  
250 mutant cell models (Supplementary Fig. S9G).

251 We recently reported CCCTC-binding factor (CTCF) motif as one of the top  
252 enriched motifs in unique *ESR1* mutant-regulated accessible genomic regions<sup>38</sup>. To  
253 determine whether CTCF has a role in the epigenetic regulation of BCK, we  
254 developed a CTCF gene signature by identification of the top 100 differentially  
255 expressed genes before and after CTCF knockdown in MCF7<sup>52</sup> (Supplementary  
256 Table S1). The positively correlated CTCF signature (i.e. using genes that were  
257 repressed after CTCF knockdown) was significantly enriched in both MCF7 *ESR1*  
258 mutant cells (Fig. 4A) and metastatic tumors (Fig. 4B) compared to their WT  
259 counterparts, whereas E2 stimulation had no effect (Fig. 4A). CTCF is a multimodal  
260 epigenetic regulator in breast cancer<sup>53</sup>, in part through generating boundaries of

261 topological associating domains (TADs) and guiding of DNA self-interaction<sup>54</sup>.  
262 Mapping the genomic occupancy of CTCF and three other cohesion complex  
263 members (RAD21, STAG1 and SMC1A) in MCF7 cells<sup>55-57</sup> (Fig. 4C) identified five  
264 putative TAD boundaries at the *KRT14/16/17* (Fig. 4D) loci and three at the  
265 *KRT5/6A/6B* (Supplementary Fig. S10A) loci. Integration of an additional MCF7  
266 CTCF ChIA-PET dataset<sup>58</sup> showed that a strong chromatin loop is predicted to span  
267 the *KRT14/16/17* genes, further supported by the pattern of convergent CTCF motif  
268 orientations at the predicted TAD boundaries (Fig. 4C). Since the *KRT5/6A/6B* locus  
269 did not harbor strong chromatin loops (>3 linkages), we focused our further analysis  
270 on the *KRT14/16/17* locus.

271       ChIP revealed strong enrichment of CTCF binding at the base of the  
272 chromatin loops of the *KRT14/16/17* locus in *ESR1* mutant cells, however there was  
273 a lack of E2 regulation (Fig. 4E). Decreasing CTCF levels led to increased  
274 expression of *KRT14*, *KRT16* and *KRT17* mRNA levels in *ESR1* mutant cells,  
275 potentially reflecting a role for CTCF as “classical” insulator, suppressing high  
276 expression of these BCKs through the identified super enhancer at the *KRT14*,  
277 *KRT16* locus (Figure 4F). Given identification of progesterone receptor (PR) binding  
278 sites within this super enhancer, PR’s previously identified role in regulating *KRT5*  
279 expression in luminal breast cancer cells<sup>47,59</sup>, and finally its overexpression in  
280 multiple *ESR1* mutant cell models<sup>25-27,60</sup> (Supplementary Fig. S10C and S10D), we  
281 tested whether PR regulates *KRT14/16/17* expression.

282       PR ChIP-seq revealed a ligand-inducible PR binding sites in MCF7 cells  
283 approximately 32kb upstream of the *KRT14/16/17* loop region<sup>61</sup> (Fig. 4F). This PR

284 binding site overlapped with a curated super-enhancer in MCF7 cells<sup>62</sup>, which was  
285 additionally supported by strong active histone modifications (Fig. S9). Knockdown  
286 of PR partially rescued the increased expression of *KRT14*, *16* and *17* in both *ESR1*  
287 mutants (Fig. 4G and Supplementary Fig. S10E). We also observed a similar rescue  
288 effect for *KRT5* (Supplementary Fig. S10E), consistent with previous studies<sup>59</sup>.  
289 Furthermore, both PR agonist (P4) and antagonist (RU486) treatment increased  
290 *KRT5*, *16* and *17* expression in Y537S *ESR1* mutant cells, while only RU486  
291 triggered *KRT5* and *KRT16* expression in D538G mutant (Fig. 4H and  
292 Supplementary Fig. S10F). The marked induction effect of RU486, a PR antagonist,  
293 is likely due to its previously reported partial agonism via recruitment of  
294 coactivators<sup>63</sup>. The RU486-induced CK5 and CK16 increase was further examined  
295 by IF, where CK5 (Supplementary Fig. S10G) and CK16 (Fig. 4I and 4J) positive  
296 cells increased from 1% to 5%. Of note, CK17 positive cells were not increased by  
297 RU486 treatment (Supplementary Fig. S10G), suggesting translational efficiency  
298 differences between different BCK subtypes. Together, these data demonstrated  
299 that elevated PR expression in *ESR1* mutant cells was essential for BCKs induction,  
300 and this was possibly due to an orchestration with a super enhancer which is  
301 accessible to regulate *KRT14/16/17* genes via the CTCF-driven chromatin loop.

302

### 303 **Enhanced immune activation, associated with S100A8-S100A9 secretion and** 304 **signaling in *ESR1* mutant tumors**

305 Finally, we investigated whether the increased expression of basal genes in  
306 *ESR1* mutant tumors confers basal-like features and potentially novel therapeutic

307 vulnerabilities. To identify basal cytokeratin-associated pathways enriched in ER  
308 mutant tumors, we at first identified ER+ tumors with the top and bottom quantile of  
309 BCK gene enrichment and then computed hallmark pathways differentially enriched  
310 between these two groups (Supplementary Fig. S11A). Intersection of these BCKs-  
311 associated pathways with those enriched in *ESR1* mutant metastases uncovered  
312 seven shared molecular functions, the top four of which are all related to immune  
313 responses (Fig. 5A, Supplementary Fig. S11B, S11C and Supplementary Table S7).  
314 An orthogonal approach - bioinformatic evaluation using ESTIMATE<sup>64</sup> - confirmed  
315 enhanced immune activation in BCK-high vs BCK-low ER+ tumors albeit still lower  
316 than in basal tumors (Fig. 5B). In addition, BCK-high tumors displayed higher  
317 lymphocyte and leukocyte fractions according to a recent biospecimens report<sup>65</sup> (Fig.  
318 5C), and higher *PDCD1* mRNA levels (Supplementary Fig. S11D). Intriguingly,  
319 patients with BCK-high ER+ tumors experience improved outcomes (Fig. 5D), and  
320 although entirely speculative at this point in time, one could hypothesize that this  
321 might be due to increased anti-tumor immune activation.

322         Similar to BCK-high ER+ tumors, *ESR1* mutant metastatic tumors exhibited  
323 higher immune scores compared to those with *ESR1* WT (Fig. 5E). Immune cell  
324 subtype deconvolution<sup>66,67</sup> revealed significantly higher CD8+ T, NK and dendritic  
325 cells, along with macrophages in *ESR1* mutant tumors. Basal breast cancers harbor  
326 high immune infiltrations at least in part due to higher tumor mutation burden  
327 (TMBs)<sup>68</sup>, however, we did not detect higher TMB in BCK-high vs low ER+ tumors  
328 (Supplementary Fig. S11E).

329 To understand which factors might contribute to immune activation in *ESR1*  
330 mutant and BCK-high ER+ tumors, we compared gene expression of major immune  
331 genes derived from ESTIMATE<sup>69</sup> (n=141) between *ESR1* mutant and WT tumors,  
332 and BCK-high vs BCK-low ER+ tumors. This analysis identified S100A8 and S100A9  
333 as the two top consistently increased immune-related genes (Fig. 6A), and this  
334 overexpression was also seen in MCF7 *ESR1* mutant cell models (Supplementary  
335 Fig. S11F). S100A8 and S100A9 are pro-inflammatory cytokines that form  
336 heterodimers and play crucial roles in shaping immune landscapes<sup>45,46</sup>. As  
337 expected, S100A8-A9 expression correlated positively with immune scores in ER+  
338 tumors (Fig. 6B). BCKs levels failed to differentiate immune scores in ER+ tumors  
339 among the subset of tumors exhibit high S100A8-A9 (Fig. 6B). S100A8-A9 are  
340 secreted proteins and function as heterodimers. To confirm S100A8-A9 protein  
341 overexpression, we measured S100A8-A9 heterodimer levels in plasma samples  
342 from patients with *ESR1* WT (n=7) and mutant (n=11) tumors (Supplementary Table  
343 S8) (Fig. 6C). This analysis revealed significantly higher circulatory S100A8-A9  
344 heterodimers concentrations in plasma from patients with *ESR1* mutations (Fig. 6D).

345 S100A8-A9 heterodimer mainly stimulates downstream cascades through two  
346 receptors: toll-like receptor 4 (TLR4) and receptor for advanced glycation end  
347 products (RAGE), and both of them are widely reported to impact cancer immunity.  
348 A further gene set variation analysis in WCRC/DFCI primary-matched paired  
349 metastatic samples revealed consistent enrichment of both pathways in *ESR1*  
350 mutant tumors (Fig. 6E, Supplementary Table S1), suggesting both TLR4 and RAGE  
351 signaling are hyperactive in *ESR1* mutant tumors.



352 To further elucidate the specific cell-cell communication by S100A8/S100A9  
353 signaling, we analyzed RAGE and TLR4 signaling via measuring ligand and receptor  
354 expression in different cell types using single-cell RNA-seq data from two breast  
355 cancer metastases. Highest expression of *S100A8/S100A9* was seen in epithelial  
356 cells, followed by fibroblast and macrophages. In contrast, TLR4 and AGER (RAGE)  
357 showed low expression in the epithelial cells, but instead were widely expressed in  
358 the stroma, especially in fibroblasts and macrophages. In general, AGER displayed  
359 lower expression levels in all cell types compared to TLR4 (Fig. 6F and 6G).

360 Taken together, these data support the concept that the increase in basal-  
361 ness of *ESR1* mutant tumors is associated with immune activation, in part facilitated  
362 by the paracrine S100A8/A9-TLR4 signaling.

## 363 Discussion

364 Recurrence of ER+ breast cancer causes over 24,000 deaths each year in  
365 the US alone. Given that *ESR1* mutation occur in as many as 20-30% of metastatic  
366 recurrences, it is imperative to identify therapeutic vulnerabilities through dissecting  
367 mechanisms of action. In this study we have uncovered a previously unrecognized  
368 plasticity of *ESR1* mutant cells, reflected by enrichment of basal subtype genes in  
369 *ESR1* mutant tumors and in particular a gain of BCK expression, resulting from  
370 epigenetic reprogramming of a mutant ER-specific PR-linked chromatin loop. This  
371 molecular evolution, i.e. an increase of basal-like feature in the *ESR1* mutant tumors  
372 was associated with immune activation including enhanced S100A8/A9-TLR4  
373 signaling (Fig 7).

374 Increased plasticity of tumors has previously been shown to be associated  
375 with tumor initiation and progression<sup>21,46,70-72</sup>. PAM50 intrinsic subtype switching has  
376 been described to occur in as many as 40% of breast cancer metastases<sup>20</sup>. Here we  
377 show that *ESR1* mutant cells gain basal-ness, and a similar observation was  
378 recently reported by Gu et al.<sup>73</sup> showing a luminal to basal switch in MCF7 *ESR1*  
379 Y537S CRISPR cells compared to parental cells. However, luminal to basal subtype  
380 switching is rare in breast cancer<sup>20</sup> and we have previously reported on clinically  
381 relevant gene expression changes in brain metastases (increased in *HER2* gene  
382 expression) without clear subtype switching<sup>18</sup>. These results are in line with the  
383 increasing appreciation of the molecular subtypes being on a continuum rather than  
384 representing discrete stages. Of note, we did not observe a similar gain of basal-  
385 ness in a series of *ESR1* wildtype endocrine resistant *in vitro* models, with the

386 exception being a study revealing a “luminal-to-basal” switch in an estradiol-deprived  
387 T47D xenograft derived cell line, indicating a potential role for the microenvironment  
388 in mediating a similar switch in ER wildtype tumors<sup>74</sup>.

389 We propose that the observed *ESR1* mutant-cancer cell state  
390 interconversions are of potential clinical relevance due to increased stromal immune  
391 activation associated with the induction of BCK. Using *in silico* gene expression,  
392 pathway analyses and pathology information, we observed increased activation of a  
393 number of immune-related pathways including S100A8/S100A9-TLR4 signaling and  
394 increased lymphocytic infiltration. S100A8/S100A9 heterodimers exhibit pro-  
395 inflammatory properties in different contexts in breast cancer<sup>75,76</sup>, are associated  
396 with poor prognosis in multiple cancer types<sup>36</sup> including breast cancer<sup>77</sup>, and  
397 blockade of their activity improves survival<sup>78</sup>. We observed increased  
398 S100A8/S100A9 levels in blood from patients with *ESR1* mutant tumors but given  
399 complexity of tumor-cell intrinsic and extrinsic roles of the inflammatory mediators  
400 and their receptors (also supported by our single cell sequencing analysis) additional  
401 work is needed to understand if and how they contribute to tumor progression in  
402 patients with ER mutant tumors. This should include an analysis of MDSC in this  
403 setting since they have been described to play an important role in S100A8/A9  
404 function<sup>76,79</sup>. This is also supported by our recent studies showing an enrichment of  
405 immune-suppressive macrophages in ER mutant tumors, along with increased  
406 expression of interferon regulated genes<sup>80</sup>. Together, these data imply opportunities  
407 for immune therapies for patients with ER mutant tumors that should be analyzed  
408 further.

409 We and others<sup>26,27,38</sup> previously identified genes that have altered expression  
410 in *ESR1* mutant cells but are not E2 regulated in WT cells. Here, all six BCK belong  
411 to this group of novel, gain-of-function target genes. BCK are not regulated as a  
412 result of ligand-mimicking nor *de novo* transactivation by mutant ER, and their  
413 expression is strongly and negatively correlated with ER levels. A similar correlation  
414 was also observed with P4-induced CK5+ luminal breast cancer cells displaying low  
415 ER and PR levels<sup>59</sup>. One possible explanation is that ER, regardless of its liganded  
416 status or genotype, serves as a direct epigenetic suppressor that represses BCK  
417 expression to maintain luminal identity. For example, it has been shown that ER  
418 silences basal, EMT and stem cell related genes by recruiting pivotal methyl-  
419 transferases like EZH2 and DNMTs to reshape the DNA and histone methylation  
420 landscape<sup>81</sup>. More studies are required to further elucidate the regulatory network  
421 between ER and BCKs. Given bi-directional interactions between tumor and stromal  
422 cells in BCK regulation, it will be important to perform future studies in improved  
423 model systems such as those recently described for analysis of complex regulation  
424 of CK14 expression and function<sup>82</sup>.

425 Assessment of BCK expression revealed that a 50-fold increase in mRNA  
426 was reflected in only ~1% cells being positive for BCK protein. This finding is  
427 consistent with a previous study showing that P4 stimulation of breast cancer cells  
428 caused a 100-fold induction of CK5 promoter activation ultimately translating to 1-  
429 10% of cells positive for CK5 protein<sup>59</sup>. In addition, discordance between mRNA and  
430 protein of CK7 and CK14 in breast cancer tissue has been documented<sup>83</sup>. It is  
431 possible that BCK protein translation in luminal cells is aberrant, resulting in poorly

432 localized or transported protein, consistent with our detection of BCK protein foci  
433 rather than the broad distribution pattern over full cytoskeleton similar to what has  
434 been previously reported for example for formation of CK17 foci. The discordance in  
435 mRNA and protein expression may be due to the cell heterogeneity, with individual  
436 cells having high mRNA and protein compared to the negative population, potentially  
437 due to heterogenous expression of miRNAs regulating BCK expression<sup>84</sup>. These  
438 BCKs positive cells might be pre-selected by multiple genetic and epigenetic cues  
439 including but not limited to low ER expression and chromatin loop formation as  
440 identified in our study. The discordance between mRNA and protein expression may  
441 also help to explain differences in prognosis using mRNA expression profiling like in  
442 our study vs IHC in previous studies<sup>85,86</sup>.

443         We provide evidence to support BCK as emerging biomarkers of *ESR1*  
444 mutant breast cancer and its prognosis, yet their direct functional impact remains  
445 ambiguous. CK14 positive cells typically lead collective invasion across major  
446 subtypes of breast cancer cells<sup>87</sup>, and this is in line with previously identified  
447 enhanced cell migration in *ESR1* mutant cells<sup>88</sup>. In addition, as previously described,  
448 CK5 positive luminal cells acquire stem-like properties and chemotherapy  
449 resistance<sup>47,59</sup>. Importantly, we found several other consistently increased basal  
450 marker genes such as interferon-alpha inducible protein 27 (IFI27). Previous studies  
451 have reported a role of IFI27 in regulating innate immunity in breast cancer<sup>89</sup> and  
452 cisplatin resistance in gastric cancer<sup>36</sup>. Thus, the “basal-ness” shift might confer  
453 several broad functional alterations to *ESR1* mutant tumors.

454 We identified a PR-orchestrated TAD at the *KRT14/16/17* genomic locus in  
455 *ESR1* mutant cells, and we propose that the simultaneous generation of a *de novo*  
456 CTCF loop and ER ligand-independent PR overexpression is necessary for  
457 *KRT14/16/17* in *ESR1* mutant cells. Intriguingly, knockdown of CTCF selectively  
458 increased *KRT14/16/17* mRNA levels whereas knockdown of PR blocked their  
459 induction in *ESR1* mutant cells. This unexpected discrepancy may highlight that  
460 CTCF binding may simultaneously serve as a transcriptional insulator to restrict  
461 *KRT14/16/17* in an inactive compartment<sup>53,90</sup>. Importantly, data indicates that CTCF  
462 knockdown alone is not sufficient to eliminate TAD but instead promotes the  
463 formation of new chromatin interactions that alter gene expression<sup>91</sup>. We also  
464 unexpectedly found that both PR agonist P4 and PR antagonist RU486 elevated  
465 BCK expression, which was inconsistent with previous reported findings where P4  
466 and RU486 exhibited opposite effects in regulating CK5<sup>59</sup>. Given RU486 is well-  
467 characterized for its partial agonism, it is possible that *ESR1* mutant cells uniquely  
468 express a particular strong PR coactivator that confers the partial agonism of RU486  
469 in this context. Another possibility is that RU486 alternatively stimulates other  
470 nuclear receptors such as glucocorticoid<sup>85,92</sup> or potentially even androgen receptor<sup>93</sup>  
471 to reprogram BCKs expression. The reversed PR pharmacological response in  
472 *ESR1* mutant cells is intriguing and warrants future investigation.

473 Our study discovered a unique aspect of *ESR1* mutant cells and addressed  
474 the underlying mechanisms as well as its clinical relevance, albeit with some  
475 remaining limitations, such as limited numbers of clinical samples due to inherent  
476 difficulties of obtaining metastatic tissues. The enhanced immune infiltration requires

477 additional validation by TIL counting on *ESR1* mutant tumor sections. Confirmation  
478 and studies in *in vivo* models should be included into future studies. Our preliminary  
479 analysis in a *ESR1* Y541S (mouse ortholog of Y537S mutation) knockin mouse  
480 model showed overexpression of BCK at RNA and protein level in mammary  
481 tumors<sup>94</sup>. And finally, the *in silico* prediction of enhanced CTCF-driven chromatin  
482 loop at the basal cytokeratin gene locus requires confirmation by orthogonal  
483 approaches, such as chromosome conformation capture. Nonetheless, our study  
484 serves as a robust pre-clinical report uncovering mechanistic insights into *ESR1*  
485 mutations and their roles in conferring basal-like feature to ER+ breast cancer and  
486 implicates the immune therapeutic vulnerabilities to this subset of patients.

## 487 **Materials and methods**

488 Additional details are provided in the Supplementary Materials and Methods  
489 section.

## 490 **Human tissue and blood studies**

491 51 paired primary matched metastatic samples were from DFCI (n=15) and  
492 our Women's Cancer Research Center (WCRC) (n=36) cohorts as previously  
493 reported<sup>95,96</sup>. For all WCRC metastatic samples, *ESR1* mutations status were called  
494 from RNA-sequencing. For bone/brain/GI metastatic lesions, *ESR1* mutations status  
495 were additionally examined using droplet digital PCR for Y537S/C/N and D538G  
496 mutations in *ESR1* LBD region as previously reported<sup>97</sup>. For DFCI cohort, *ESR1*  
497 mutations were all called from matched whole exome sequencing<sup>98</sup>.

498 For the study of patients' blood, all patients provided written informed consent  
499 and all procedures were approved by the University of Pittsburgh Institutional  
500 Review Board (PRO17080172). 18 patients diagnosed with late-stage metastatic  
501 ER+ breast cancer were recruited. Procedure to identify hotspot *ESR1* mutations  
502 has been previously described by us<sup>99</sup>.

## 503 **Cell culture**

504 Establishments of rAAV-edited (Park lab)<sup>27</sup>, CRISPR-Cas9-edited (Gertz<sup>38</sup>  
505 and Ali<sup>25</sup> lab) and CRISPR-Cas9-edited T47D cells<sup>27</sup> were reported previously.  
506 ZR75-1 (CRL-1500), MDA-MB-134-VI (HTB-23), MDA-MB-330 (HTB-127) and MDA-  
507 MB-468 (HTB-132) were obtained from the ATCC. Development of BCK4 cells has  
508 been previously reported<sup>100</sup>.



## 509 **S100A8/S100A9 heterodimer ELISA**

510 Human S100A8/S100A9 heterodimer amounts in human plasma samples  
511 were quantified using S100A8/S100A9 heterodimer Quantikine ELISA kit (R&D  
512 System, DS8900) following the manufacture protocol. All plasma samples were first  
513 diluted in calibration buffer with 1:50 ratio and loaded into antibody-coated plate.

## 514 **Chromatin-immunoprecipitation (ChIP) and sequencing analysis**

515 ChIP was performed as previously described<sup>51</sup>. ChIP-seq reads were aligned  
516 to hg38 genome assembly using Bowtie 2.0<sup>101</sup>, and peaks were called using  
517 MACS2.0 with p value below 10E-5<sup>102</sup>. We used DiffBind package<sup>103</sup> to perform  
518 principle component analysis, identify differentially expressed binding sites and  
519 analyze intersection ratios with other data sets. Heatmaps and intensity plots for  
520 binding peaks were visualized by EaSeq. Annotation of genes at peak proximity was  
521 conducted using ChIPseeker<sup>104</sup>, taking the promoter region as +/- 3000 bp of the  
522 transcriptional start site (TSS) and 50kb as peak flank distance.

## 523 **RNA sequencing analysis**

524 RNA sequencing data sets were analyzed using R version 3.6.1. Log2  
525 (TPM+1) values were used for the RNA-seq of Oesterreich *ESR1* mutant cell models  
526 and TMM normalized Log2(CPM+1) values were used for Gertz RNA-seq data.  
527 TCGA reads were reprocessed using Salmon v0.14.1<sup>105</sup> and Log2 (TPM+1) values  
528 were used. For the METABRIC data set, normalized probe intensity values were  
529 obtained from Synapse. For genes with multiple probes, probes with the highest  
530 inter-quartile range (IQR) were selected to represent the gene. For pan-breast  
531 cancer cell line transcriptomic clustering, 97 breast cancer cell line RNA-seq data

532 were reprocessed using Salmon and merged from three studies<sup>34-36</sup>, batch effects  
533 were removed using “removeBatchEffect” function of “limma<sup>106</sup>” package. Gene set  
534 variation analysis was performed using “GSVA” package<sup>107</sup>. Survival comparisons  
535 were processed using “survival” and “survminer” packages<sup>108</sup> using Cox  
536 Proportional-Hazards model and log-rank test. Data visualizations were performed  
537 using “ggpubr<sup>109</sup>”, “VennDiagram<sup>110</sup>” and “plot3D<sup>111</sup>”.

538 For the single cell RNA seq analysis, two fresh bilateral bone metastases  
539 (BoMs) were collected from a patient initially diagnosed with ER+ primary breast  
540 cancer, dissociated into single cells and a cell suspension with at least 70% viability  
541 was submitted for library preparation using 10X genomics chromium platform (V3.0  
542 chemistry) (Ding et al, manuscript in preparation). 6,000 cells were targeted for each  
543 BoM, and the final libraries were sequenced at a depth of 67,000 reads per cell  
544 using NOVAseq.

#### 545 **Tumor Mutation Burden Analysis**

546 Tumor mutation burden (TMB) calculation was performed as previous  
547 described<sup>112</sup>. Briefly, TCGA mutation annotation files from 982 patients were  
548 downloaded from FireBrowse and mutation subtypes were summarized using  
549 “maftool” package<sup>113</sup>. Mutations subtypes were classified into truncated (nonsense,  
550 frame-shift deletion, frame-shift insertion, splice-site) and non-truncated mutations  
551 (missense, in-frame deletion, in-frame insertion, nonstop). TMB was calculated as  
552 2X Truncating mutation numbers + non-truncating mutation numbers.

#### 553 **Generation of Gene Sets**

554 For Sorlie et al., the original set of intrinsic genes were downloaded from  
555 Stanford Genomics Breast Cancer Consortium ([http://genome-  
556 www.stanford.edu/breast\\_cancer/](http://genome-<br/>556 www.stanford.edu/breast_cancer/)). 453 genes were annotated from 553 probes.  
557 Expression of these 453 genes were examined in 33 luminal and 39 basal breast  
558 cancer cell lines. Significantly higher (FDR<0.01) intrinsic genes in basal or luminal  
559 cells were called as basal (n=75) or luminal (n=68) markers in Sorlie gene sets. For  
560 the TCGA gene set, differentially expressed genes were called between basal and  
561 luminal A or basal and luminal B ER+ tumors using raw counts. The top 200  
562 increased genes of these two comparisons were further intersected. Overlapped DE  
563 genes in basal (n=164) and luminal (n=139) tumors were called as TCGA gene sets.  
564 For CTCF gene signature establishment, a previous RNA-seq data set on MCF7  
565 cells with or without CTCF knockdown was downloaded and analyzed<sup>52</sup>, top 100  
566 downregulated genes with CTCF knockdown were used as the CTCF gene  
567 signature.

### 568 **Chromatin interaction data analysis**

569 CTCF ChIA-PET data were downloaded from GSE72816. Chromatin linkages were  
570 visualized on 3D genome browser (<http://promoter.bx.psu.edu/hi-c/>) after processed  
571 with ChIA-PET tool<sup>114</sup>. Confident TAD boundaries were defined by the colocalization  
572 of CTCF and cohesion complex subunits together with called chromatin interactions.

### 573 **Data Availability**

574 ER ChIP-seq data from MCF7 *ESR1* mutant cell model was deposited in  
575 Gene Expression Omnibus with accession number of GSE125117. MSigDB curated  
576 gene sets were downloaded from GSEA website

577 (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>). RNA-seq data and clinical  
578 information from TCGA and METABRIC were obtained from the GSE62944 and  
579 Synapse software platform (syn1688369) respectively. TCGA biospecimen immune  
580 profile data were downloaded from Saltz et al<sup>65</sup>. TCGA mutation annotation format  
581 (MAF) files and methylation data were downloaded from FireBrowse website  
582 (<http://firebrowse.org/>). Complete RNA-Seq data for the DFCI metastases samples  
583 will be published separately. RNA-Seq data from the WCRC cohorts are available at  
584 Lee-Oesterreich Lab Github repository (<https://github.com/leeoesterreich>). All the  
585 raw data and scripts are available upon request from the corresponding author.  
586 Sources of all public available data sets used in this study are summarized in  
587 Supplementary Table S10.

588

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607

#### 608 **Competing Interests**

609 The authors declare no conflict of interests.

610

#### 611 **Author Contributions**

612 Z.L., J.M.A., A.V.L. and S.O. conceived and designed the study. Z.L., Y.W., A.B. and  
613 K.D. designed, performed and analyzed experiments. Z.L., A.B., N.M.P. and K.D.  
614 performed bioinformatic analysis. J.M.A., L.M. and M.R. contributed to clinical  
615 sample collection and intellectual input. N.W. provided extended RNA-seq data set  
616 (DFCI) from clinical specimens and intellectual input. Z.L., A.V.L., S.O., C.A.S.,  
617 J.K.R., W.J.M. and J.G. contributed to data interpretation and provided additional  
618 intellectual input. L.B., S.A. and J.G. provided additional cell models for this study  
619 and intellectual input. Y.F., L.Z. and G.C.T. provided and validated biostatistical

620 approaches of all the analysis. Z.L., A.V.L. and S.O. developed the figures and the  
621 manuscript. All the authors reviewed and agreed with the contents of the manuscript.

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- 901

902 **Figure legends**

903 **Figure 1. Basal breast cancer gene sets are enriched in *ESR1* mutant breast**  
904 **cancers**

905 A) Four pairs of luminal/basal gene sets applied in this study with gene numbers  
906 specified in each set.

907 B) Venn diagram representing the overlap of genes from the basal (left) and luminal  
908 (right) gene sets. Genes overlapping in at least three gene sets are indicated.

909 C) and D) Dot plots showing GSVA score of the four pairs of basal (C) and luminal  
910 (D) gene sets enrichment in MCF7 genome-edited cell models. Scores from luminal  
911 and basal breast cancer cell lines were used as positive controls. Dunnett's test was  
912 used to compare with WT-vehicle set within each gene set. (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

913 E) and F) Box plots representing basal (E) and luminal (F) gene set enrichments in  
914 intra-patient matched paired primary-metastatic samples. Delta GSVA score for  
915 each sample was calculated by subtracting the scores of primary tumors from the  
916 matched metastatic tumors. Mann-Whitney U test was performed to compare the  
917 Delta GSVA scores between WT (N=44) or *ESR1* mutation-harboring (N=7) paired  
918 tumors. (\*  $p < 0.05$ )

919

920 **Figure 2. Overexpression of basal cytokeratins (BCK) in *ESR1* mutant breast**  
921 **cancer cells and tumors**

922 A) Correlation between basal gene fold changes (FC) in MCF7-Y537S/D538G cells  
923 (normalized to WT vehicle) and intra-patient paired mutant tumors (normalized to  
924 WT tumors) (N=634). Consistently increased or decreased genes in the two MCF7

925 mutant cells and tumors compared to their WT counterparts were highlighted in red  
926 or blue respectively, and six basal cytokeratin genes are indicated. Inconsistently  
927 changed genes among the three comparisons are labelled in black.

928 B) *KRT5/6A/6B/14/16/17* mRNA levels in MCF7 WT and *ESR1* mutant cells.  
929 Relative mRNA fold change normalized to WT cells and *RPLP0* levels measured as  
930 the internal control. Each bar represents mean  $\pm$  SD with three biological replicates.  
931 Representative results from three independent experiments are shown. Dunnett's  
932 test was used to compare BCKs expression levels between WT and mutant cells.

933 C) Representative images of immunofluorescence staining on CK5, CK16 and CK17  
934 in MCF7 WT and *ESR1* mutant cells. Regions with CK positive cells were  
935 highlighted in the magnified images. MDA-MB-468 was included as positive control.  
936 Images were taken under 20x magnification.

937 D) Quantification of percentages of CK positive cells in MCF7 WT and *ESR1* mutant  
938 cells. Each bar represents mean  $\pm$  SD from four different regions. Data shown are  
939 from one representative experiment of three independent experiments. Dunnett's  
940 test was used to compare BCKs positive cell prevalence between WT and mutant  
941 cells. (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

942

943 **Figure 3. Basal cytokeratins induction is independent of mutant ER genomic**  
944 **binding but requires low ER expression.**

945 A) Heatmap representing fold change mRNA expression (E2/veh) of six basal  
946 cytokeratins and four luminal cytokeratins in ER+ breast cancer lines from six

947 publicly available data sets (GSE89888, GSE94493, GSE108304, GSE3834,  
948 GSE38132 and GSE50693). *GREB1*, *PGR*, and *TFF1* are canonical E2-regulated  
949 genes included as positive controls.

950 B) Genomic track showing ER binding intensities at *KRT5/6A/6B* and *KRT14/16/17*  
951 loci from ER ChIP-seq data sets of MCF7 *ESR1* mutant cells. *GREB1* locus serve as  
952 a positive control.

953 C) Graphic view of Pearson correlation between expression of *ESR1* and each basal  
954 or luminal cytokeratin in ER+ breast tumors in TCGA (n=808) and METABRIC  
955 (n=1,505) cohorts. Color scale and size of dots represent correlation coefficient and  
956 significance, respectively.

957 D) qRT-PCR measurement of *ESR1*, *KRT5/6A/6B/14/16/17* mRNA levels in MCF7  
958 WT and *ESR1* mutant cells with *ESR1*siRNA knockdown for 7 days. mRNA fold  
959 change normalized to WT cells; RPLP0 levels were measured as internal control.  
960 Each bar represents mean  $\pm$  SD with three biological replicates. Data shown are  
961 representative from three independent experiments. Student's t-test was used to  
962 compare the gene expression between scramble and knockdown groups. (\*  $p < 0.05$ ,  
963 \*\*  $p < 0.01$ )

964 E) Representative images of ER, CK5, CK16 and CK17 staining in MCF7-Y537S  
965 and D538G cells. BCKs positive cells are highlighted with white arrows. Images  
966 were taken under 20x magnification.

967 F) Bar plots quantifying the ER intensities in BCKs positive (blue) and the  
968 corresponding proximal negative (red) cells from each region. Each bar represents  
969 mean  $\pm$  SD analyzed in five different regions per group from one experiment,



970 representative of three independent experiments. Paired t test was applied to  
971 compare ER intensities between BCKs positive and negative cells. (\*  $p < 0.05$ , \*\*  
972  $p < 0.01$ )

973

974 **Figure 4. Basal cytokeratins are induced via a unique PR enhancer-associated**  
975 **TAD in *ESR1* mutant cells.**

976 A) Dot plots showing enrichment levels of CTCF gene signature in MCF7 *ESR1*  
977 mutant cells. Dunnett's test was used to compare the difference. (\*\*  $p < 0.01$ )

978 B) Dot plots showing enrichment levels of CTCF gene signature in *ESR1* WT (n=44)  
979 and mutant (n=7) metastases. Mann-Whitney U test was used to compare  
980 enrichment levels in tumors. (\*  $p < 0.05$ )

981 C) Genomic track illustrating the CTCF/cohesion complex binding at *KRT14/16/17*  
982 proximal genomic region in MCF7 cells. CTCF and RAD21 ChIP-seq were  
983 downloaded from ENCODE (ENCSR560BUE and ENCSR703TNG). STAG1 and  
984 SMC1A ChIP-seq data were from GEO (GSE25021 and GSE76893). CTCF motif  
985 orientations of each peak is labelled with black arrows in the CTCF track. Y-axis  
986 represents signal intensity of each track.

987 D) CTCF-driven chromatin loops visualized using a CTCF ChIA-PET data set in  
988 MCF7 cells (GSE72816) at the 3D Genome Browser platform. Each linkage  
989 represents a chromatin loop.

990 E) Bar graphs displaying CTCF binding events measured by ChIP-qPCR at binding  
991 sites 1 and 5 illustrated in (C). CTCF binding fold enrichments were normalized to

992 the average of IgG binding. Each bar represents mean  $\pm$  SD of fold changes from  
993 three independent experiments. Pair-wise t-test on CTCF binding fold enrichment  
994 between WT and each mutant was performed. (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

995 F) PR binding under R5020 and progesterone treatments visualized based on a  
996 reported PR ChIP-seq data set in MCF7 cells (GSE68359). Y-axis represents signal  
997 intensity of each track and is adjusted to the same scale. Super enhancer range was  
998 highlighted below the genomic track.

999 G) qRT-PCR measurement of *KRT14*, *16* and *17* mRNA levels in MCF7 *ESR1* WT  
1000 and mutant cells with PGR siRNA knockdown for 7 days. mRNA fold change  
1001 normalized to WT cells; RPLP0 levels were measured as internal control. Each bar  
1002 represents mean  $\pm$  SD with three biological replicates. Data shown are  
1003 representative from three independent experiments. Student's t-test was used to  
1004 compare the gene expression between scramble and knockdown groups. (\*  $p < 0.05$ ,  
1005 \*\*  $p < 0.01$ )

1006 H) qRT-PCR measurement of *KRT5*, *16* and *17* mRNA levels in MCF7 *ESR1* WT  
1007 and mutant cells treated with 0.1% EtOH (vehicle), 100 nM P4 or 1  $\mu$ M RU486  
1008 treatment for 3 days. mRNA fold change normalized to WT cells; RPLP0 levels  
1009 were measured as internal control. Each bar represents mean  $\pm$  SD with three  
1010 biological replicates. Data shown are representative from three independent  
1011 experiments. (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

1012 I) Representative images of immunofluorescence staining of CK5 and CK16 in  
1013 MCF7 WT and *ESR1* mutant cells after 3 day treatment with 1% EthOH (vehicle) or  
1014 1  $\mu$ M RU486. Images were taken under 20x magnification.

1015 J) Quantification of the percentages of CK positive cells in MCF7 cells. Each bar  
1016 represents mean  $\pm$  SD from eight different regions combining from two independent  
1017 experiments. Student's t test was used to compare % BCK+ cells before and after  
1018 treatment. (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

1019

1020 **Figure 5. Gain of basal cytokeratin expression is associated with enhanced**  
1021 **immune activation in *ESR1* mutant tumors.**

1022 A) Venn diagrams showing the intersection of significantly enriched hallmark  
1023 pathways in three sets of comparisons: BCK-high vs low in 1) TCGA ER+ tumors  
1024 (n=202 in each group), 2) METABRIC ER+ tumors (n=376 in each group) and 3)  
1025 *ESR1* mutant (n=7) vs WT (n=44) metastatic tumors. BCKs high and low were  
1026 defined by the upper and bottom quartiles of each subset. The seven overlapping  
1027 pathways are shown in a frame, and immune-related pathways are highlighted in  
1028 red.

1029 B) Immune scores based on ESTIMATE evaluations in basal tumors (METABRIC  
1030 n=328; TCGA n=190), BCK-high (METABRIC n=376; TCGA n=202) and low  
1031 (METABRIC n=376; TCGA n=202) subsets of ER+ tumors in TCGA and  
1032 METABRIC. Definition of BCK-high and low groups were the same in (A). Mann  
1033 Whitney U test was used for comparison. (\*\*  $p < 0.01$ )

1034 C) Lymphocytes and leukocyte fractions as determined by a reported TCGA  
1035 biospecimen dataset<sup>65</sup> comparing among basal subtype tumors (n=161), TCGA ER+  
1036 BCK-high (N=163) and low (N=179) tumors. Definition of BCK-high and low groups

1037 were the same in (A). Mann Whitney U test was applied to compare the fractions  
1038 between BCK-high and low tumors. (\*\*  $p < 0.01$ )

1039 D) Kaplan-Meier plots showing the disease-specific survival (DSS) (METABRIC) and  
1040 overall survival (OS) (TCGA) comparing patients with ER+ BCKs high vs low tumors.  
1041 BCKs high and low were defined by the upper and bottom quartiles of each subset.  
1042 Censored patients were labelled in cross symbols. Log rank test was used and  
1043 hazard ratio with 95% CI were labelled.

1044 E) Immune scores based on ESTIMATE evaluations in *ESR1* mutant (n=7) and WT  
1045 metastatic (n=44) lesions. Mann Whitney U test was used for comparison. (\*  $p < 0.05$ )

1046 F) Dot plot showing the enrichment level alterations of immune cell subtypes in  
1047 *ESR1* mutant metastatic lesions using Davoli<sup>66</sup> and Tamborero<sup>67</sup> immune cell  
1048 signatures. RNA seq data from intra-patient matched *ESR1* mutant (N=7) and WT  
1049 (N=44) was used. Immune cell subtypes showing significant increase in *ESR1*  
1050 mutant tumors were labelled in red ( $p < 0.05$ ).

1051

1052 **Figure 6. Immune activation in *ESR1* mutant tumors is associated with**  
1053 **S100A8/A9-TLR4 paracrine crosstalk between epithelial and stromal cells.**

1054 A) Three-dimensional plot showing fold change (FC) expression changes of immune  
1055 genes from ESTIMATE (N=141)<sup>69</sup> comparing ER+ BCK-high vs low tumors (TCGA  
1056 and METABRIC) and intra-patient paired *ESR1* WT/mutant tumors. Consistently  
1057 increased/decreased genes in TCGA and METABRIC BCK-high tumors and *ESR1*

1058 mutant tumors were highlighted in red and blue. Inconsistently changed genes  
1059 among the three comparisons are labelled in black.

1060 B) ER+ cases with BCK-high and low quantiles were further divided by the mean  
1061 expression of S100A8 and S100A9. ESTIMATE immune scores were compared  
1062 across all four subsets (n=188 and 101 in each group of METABRIC and TCGA)  
1063 together with basal tumors (n=328 METABRIC and n=190 TCGA). Each  
1064 corresponding comparison was tested using Mann Whitney U test. (\*\*p<0.01)

1065 C) Graphical presentation of strategy to quantify and compare S100A8/9  
1066 heterodimer abundance in plasma from patients with ER+ metastatic breast cancer.

1067 D) Box plot showing S100A8/9 heterodimer concentrations in plasma from patients  
1068 with *ESR1* WT (n=7) and mutant (n=11) metastatic breast cancer. Mann Whitney U  
1069 test was utilized. (\* p<0.05)

1070 E) Comparison of TLR4 (left) and RAGE (right) signaling signature enrichments in  
1071 intra-patient matched *ESR1* mutant (N=7) and WT (N=44) cohort. Delta GSVA score  
1072 of each sample was calculated by subtracting the scores of primary tumors from the  
1073 matched metastatic tumors. Mann-Whitney U test was performed between WT and  
1074 mutant tumors. (\*\*p<0.01)

1075 F) Violin plots showing S100A8, S100A9, TLR4 and AGER expression by log<sub>2</sub>  
1076 normalized counts in different cell subtypes using single-cell RNA-seq data from two  
1077 bone metastases from a patient with ER+ breast cancer.

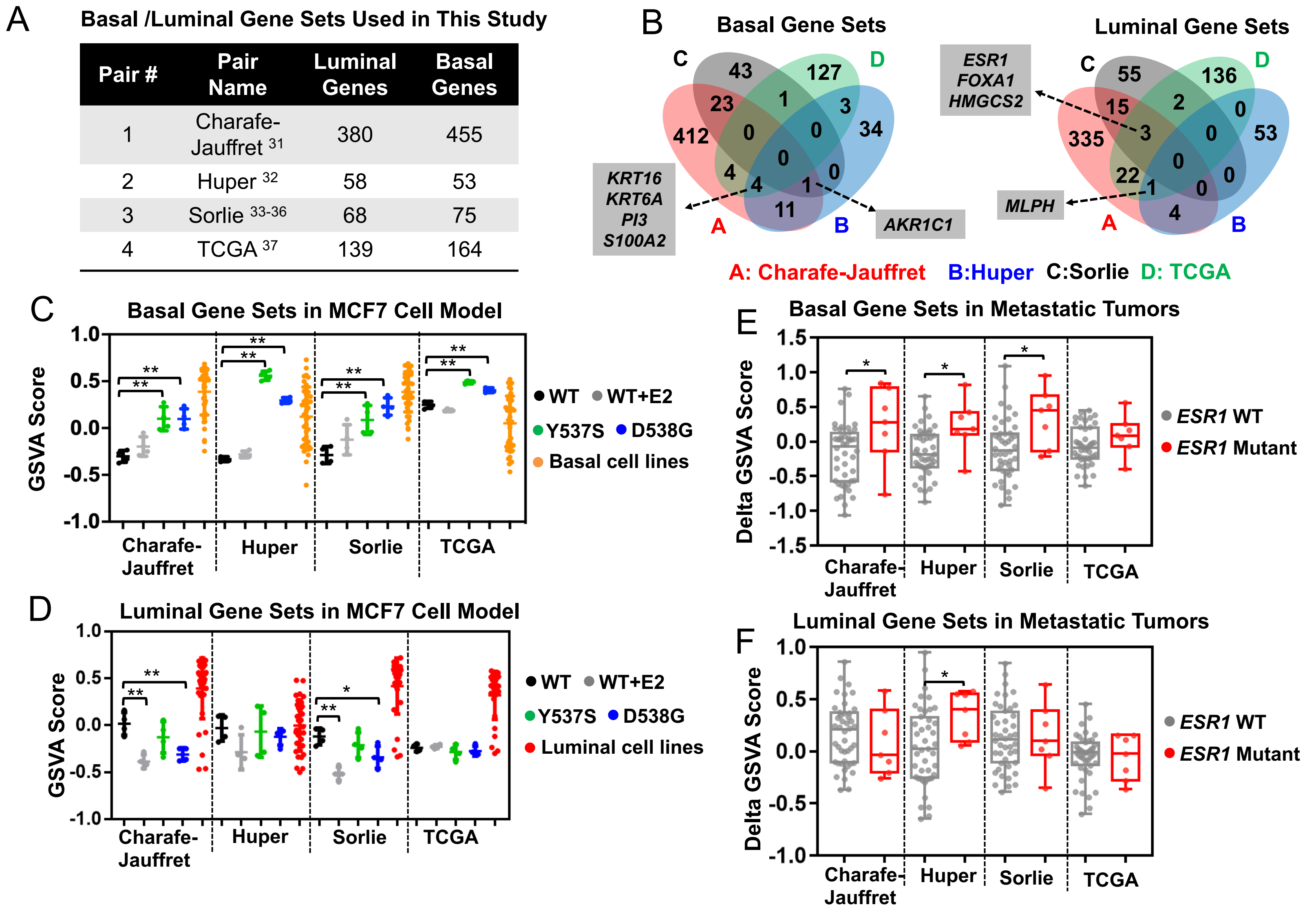
1078 G) Percent of cells expressing S100A8, S100A9, TLR4 and AGER, using single cell  
1079 RNA seq data shown in Figure 6F.

1080

1081 **Figure 7. Graphical presentation of proposed mechanisms and relevance of**  
1082 **basal cytokeratin induction in *ESR1* mutant breast cancer.**

1083 *ESR1* WT cells exhibit low basal cytokeratin expression with baseline TAD  
1084 prevalence spanning *KRT14/16/17* loci. In contrast, a minor subpopulation of *ESR1*  
1085 mutant cells exhibit strong basal cytokeratin expression, due to PR activated  
1086 enhancer at the *KRT14/16/17* gene locus-spanning TAD. Increased expression of  
1087 basal cytokeratin is associated with immune activation in *ESR1* mutant tumor similar  
1088 to that seen in basal tumors, at least in part mediated via enhanced S100A8/A9-  
1089 TLR4 paracrine crosstalk between epithelial and stromal cells, including  
1090 macrophages.

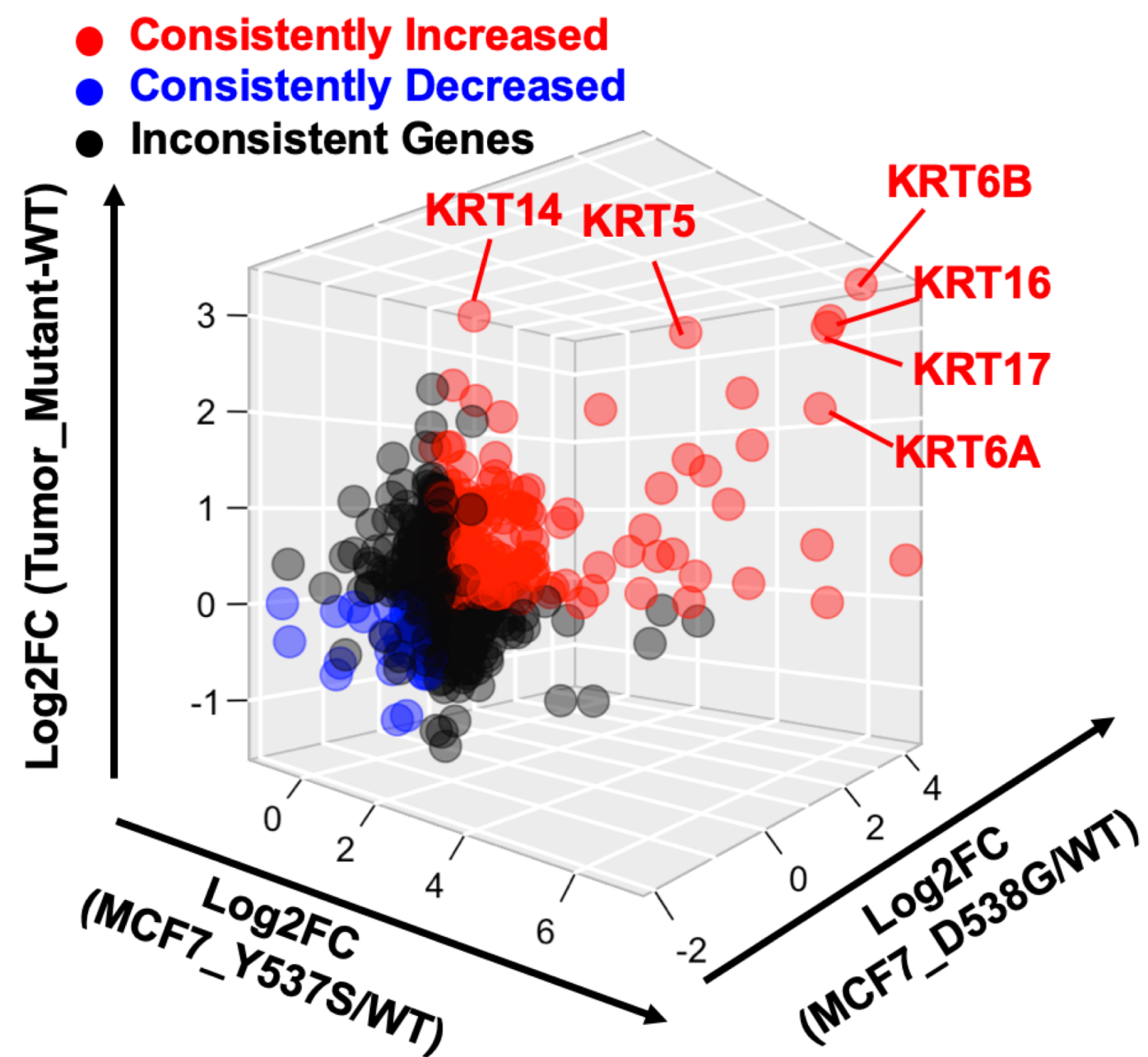
# Main Figure 1



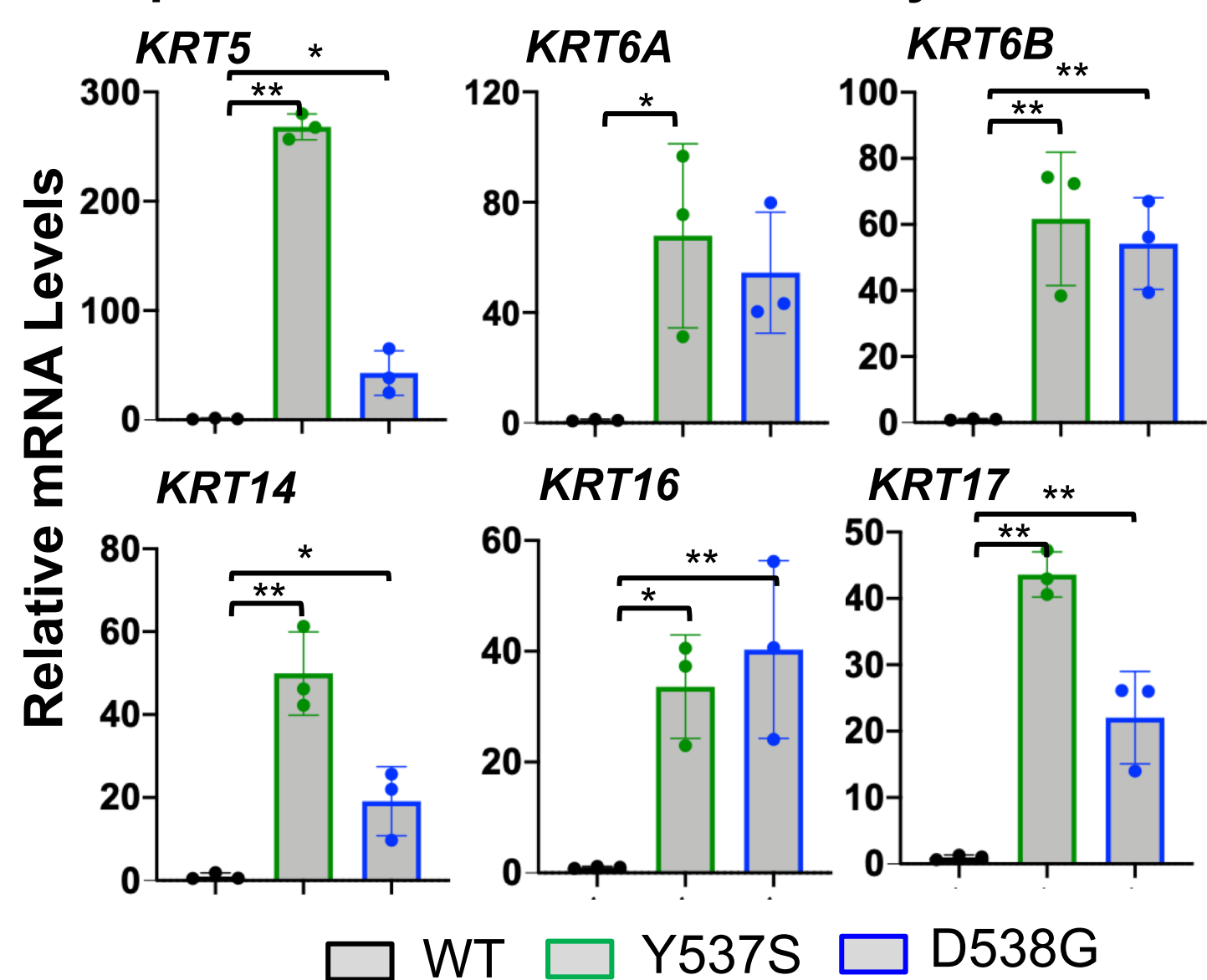
**Figure 1. Basal breast cancer gene signatures are enriched in *ESR1* mutant breast cancer**

# Main Figure 2

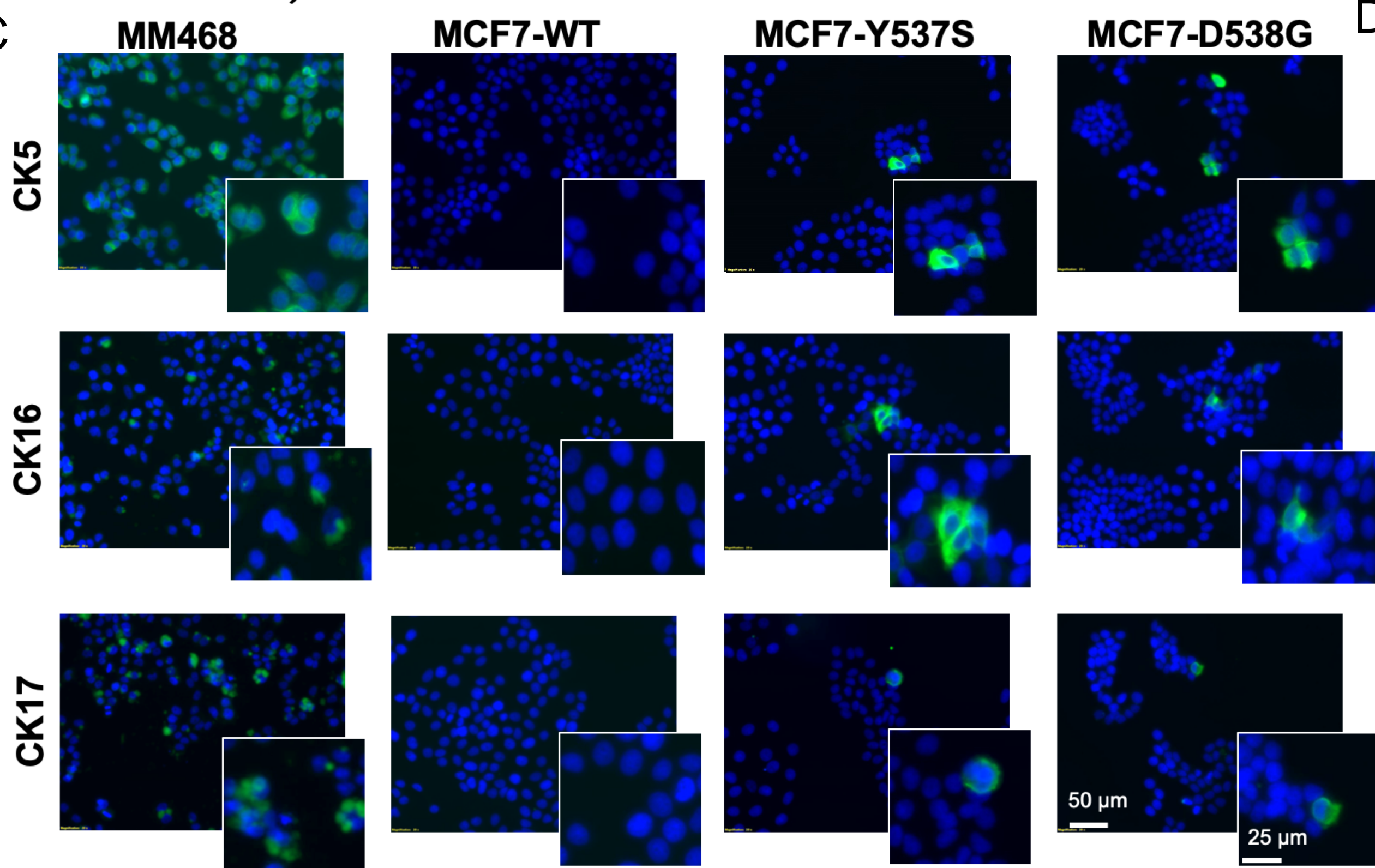
## A Fold Change Correlation of Basal Genes



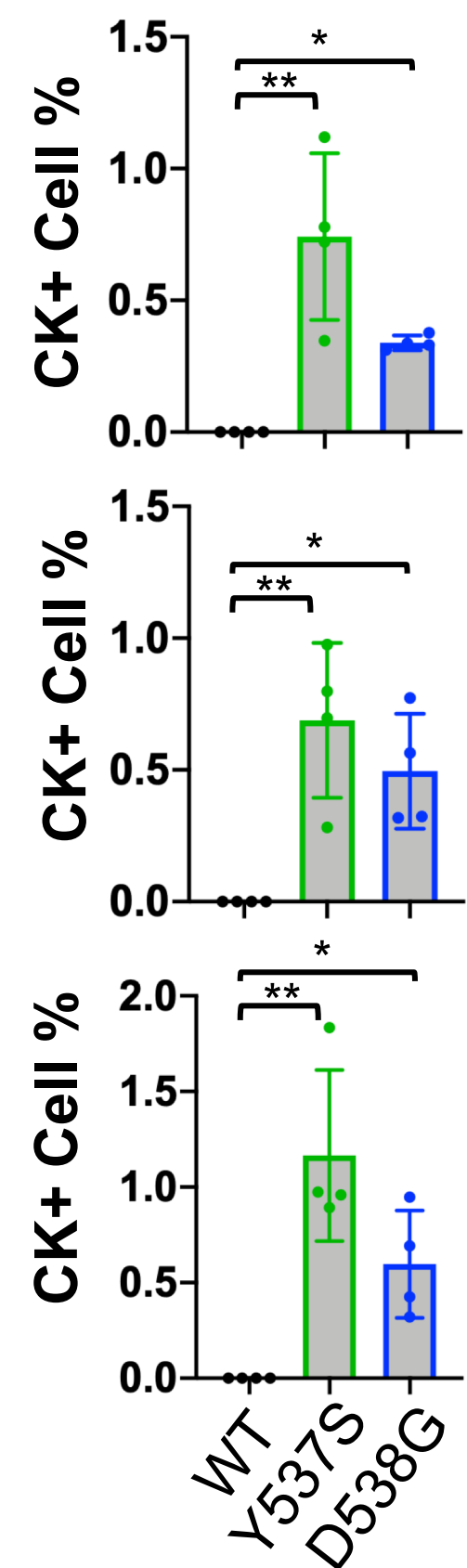
## B qRT-PCR Validation of Basal Cytokeratins



## C



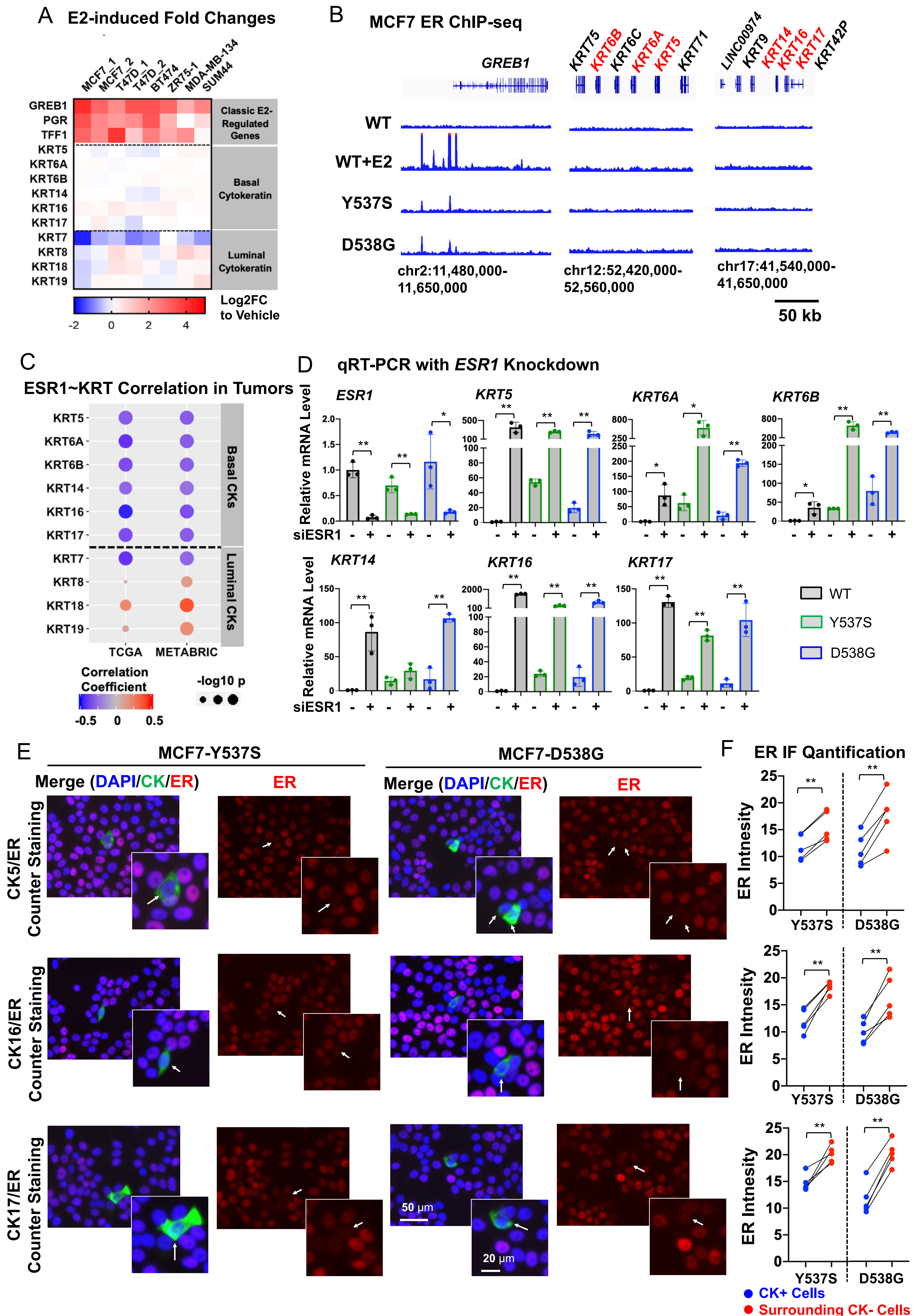
## D IF Quantification



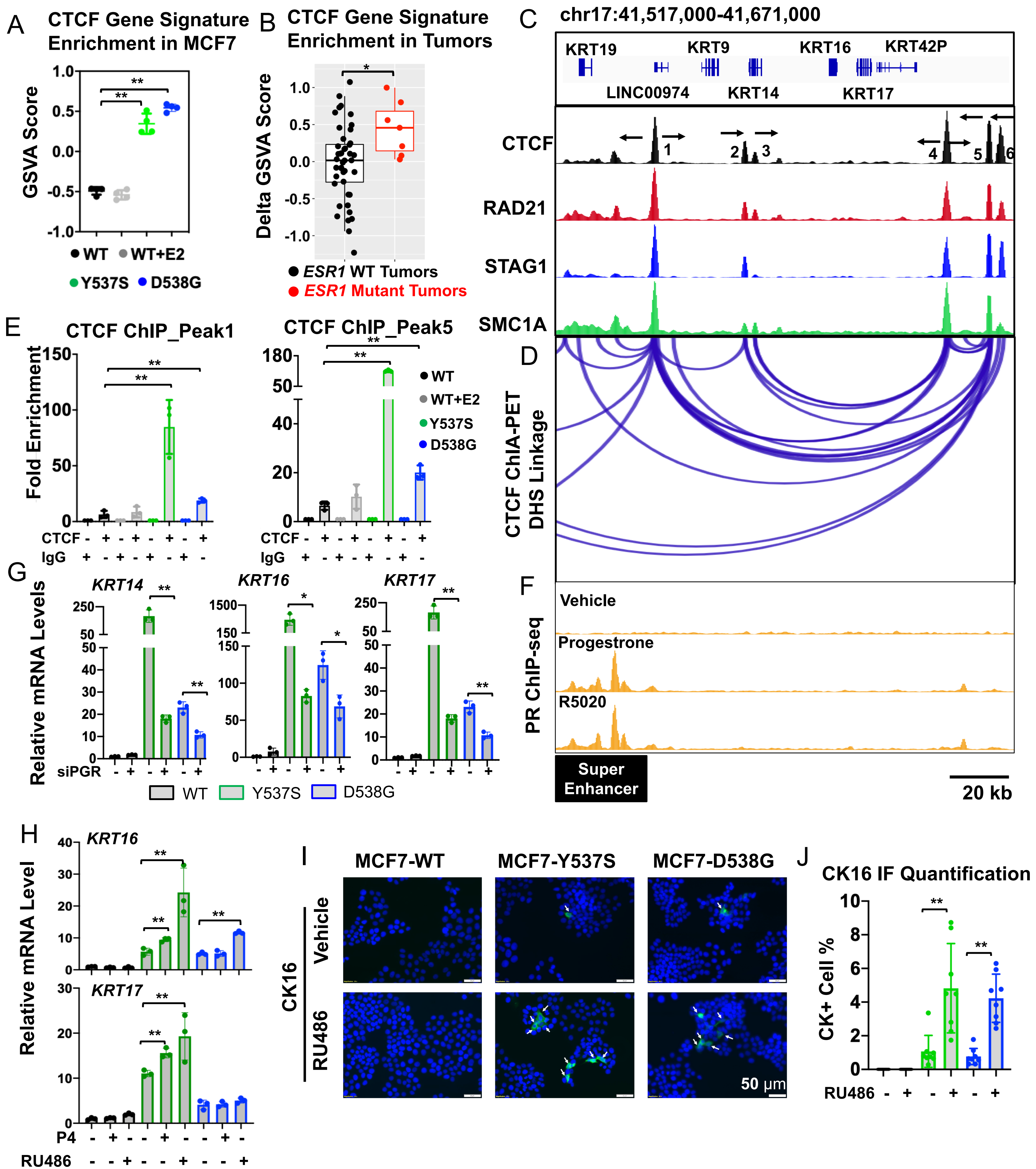
**Figure 2. Basal cytokeratins are the leading enriched basal markers in *ESR1* mutant breast cancer cells and tumors**



# Main Figure 3

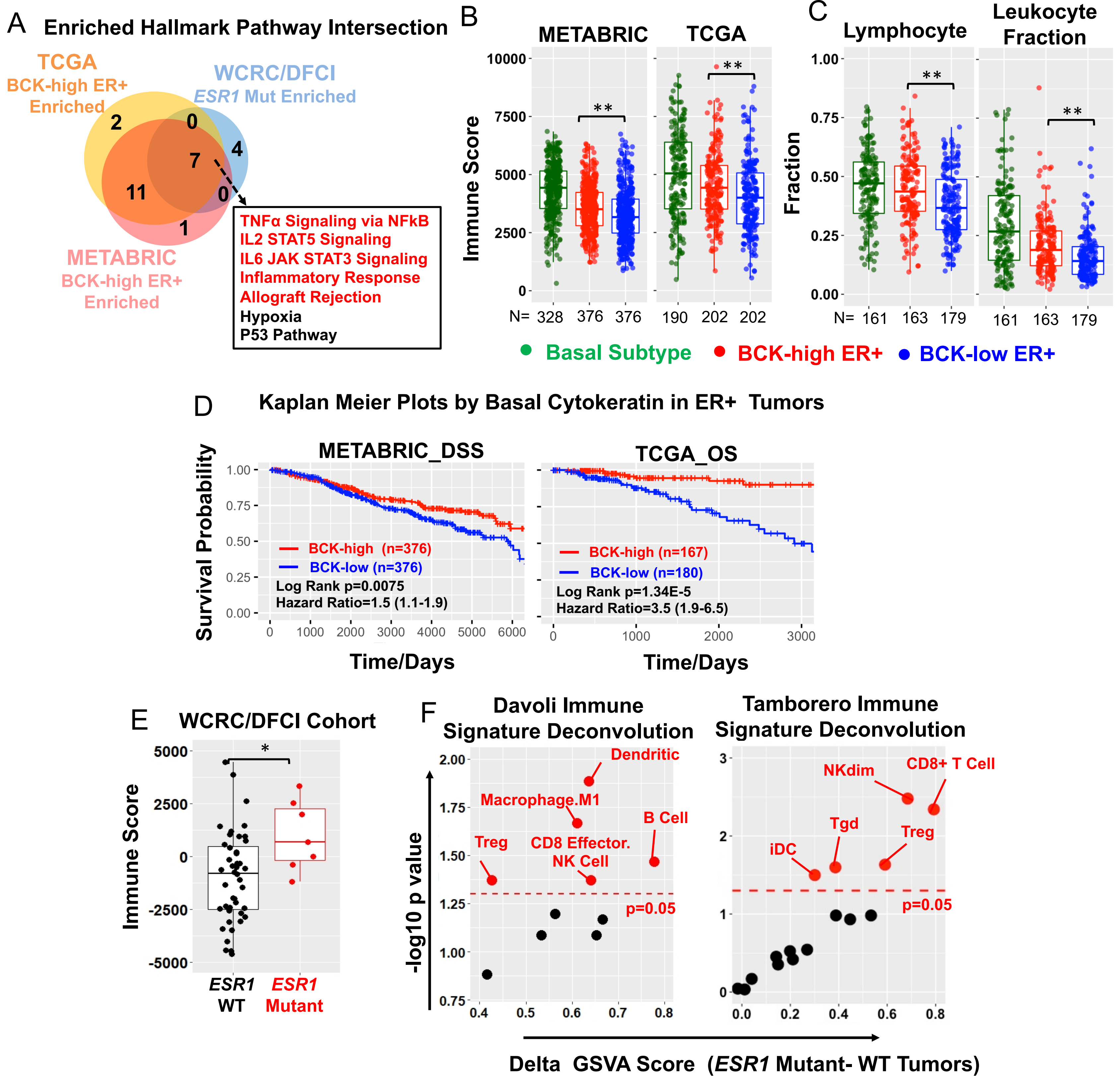


# Main Figure 4



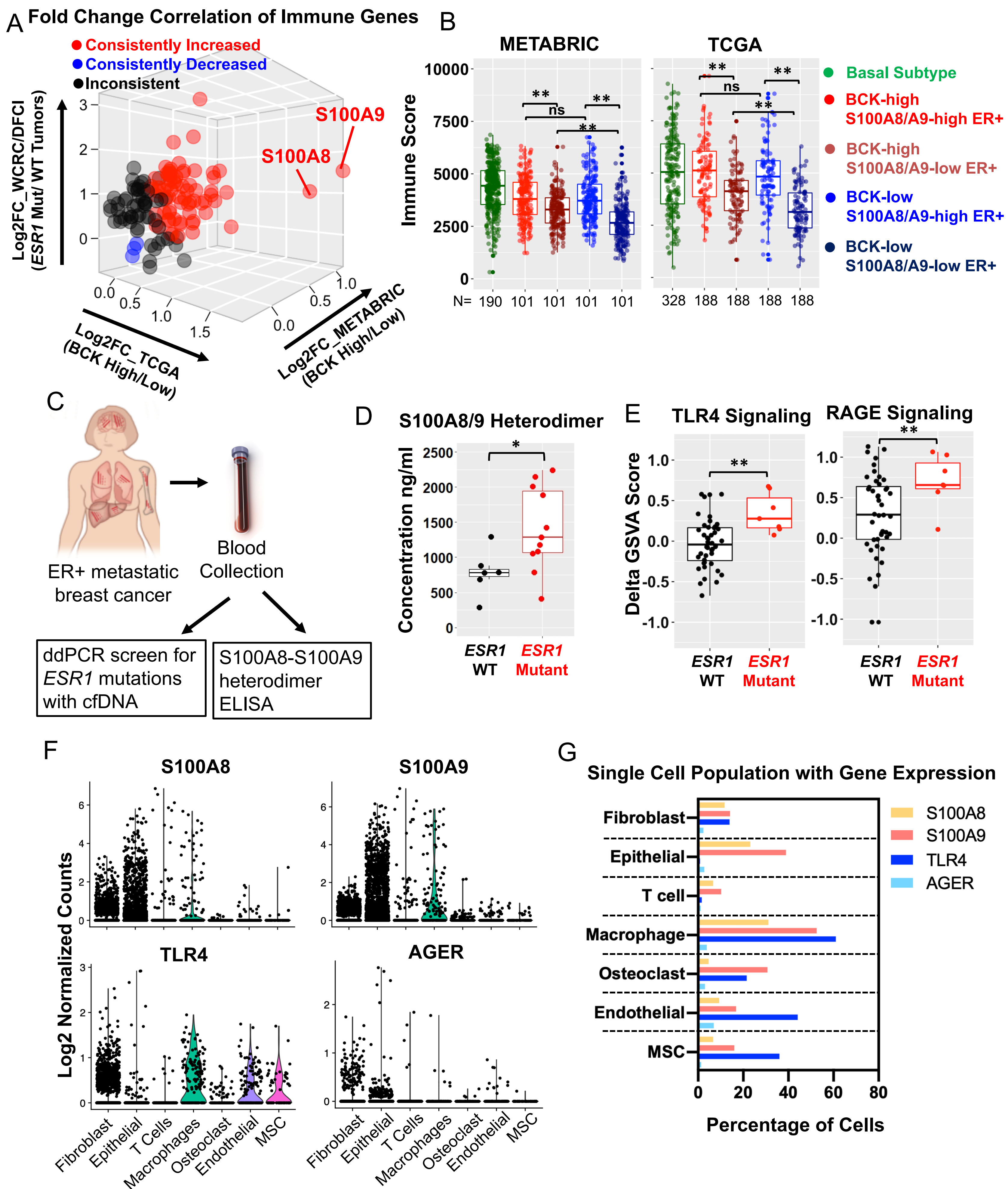
**Figure 4. Basal cytokeratins are induced via a unique PR enhancer associated TAD in *ESR1* mutant cells**

# Main Figure 5



**Figure 5. Gain of basal cytokeratins is associated with enhanced immune activation in *ESR1* mutant tumors**

# Main Figure 6



**Figure 6. Immune activation in *ESR1* mutant tumors is associated with S100A8/A9-TLR4 paracrine crosstalk between epithelial and stromal cells.**

# Main Figure 7

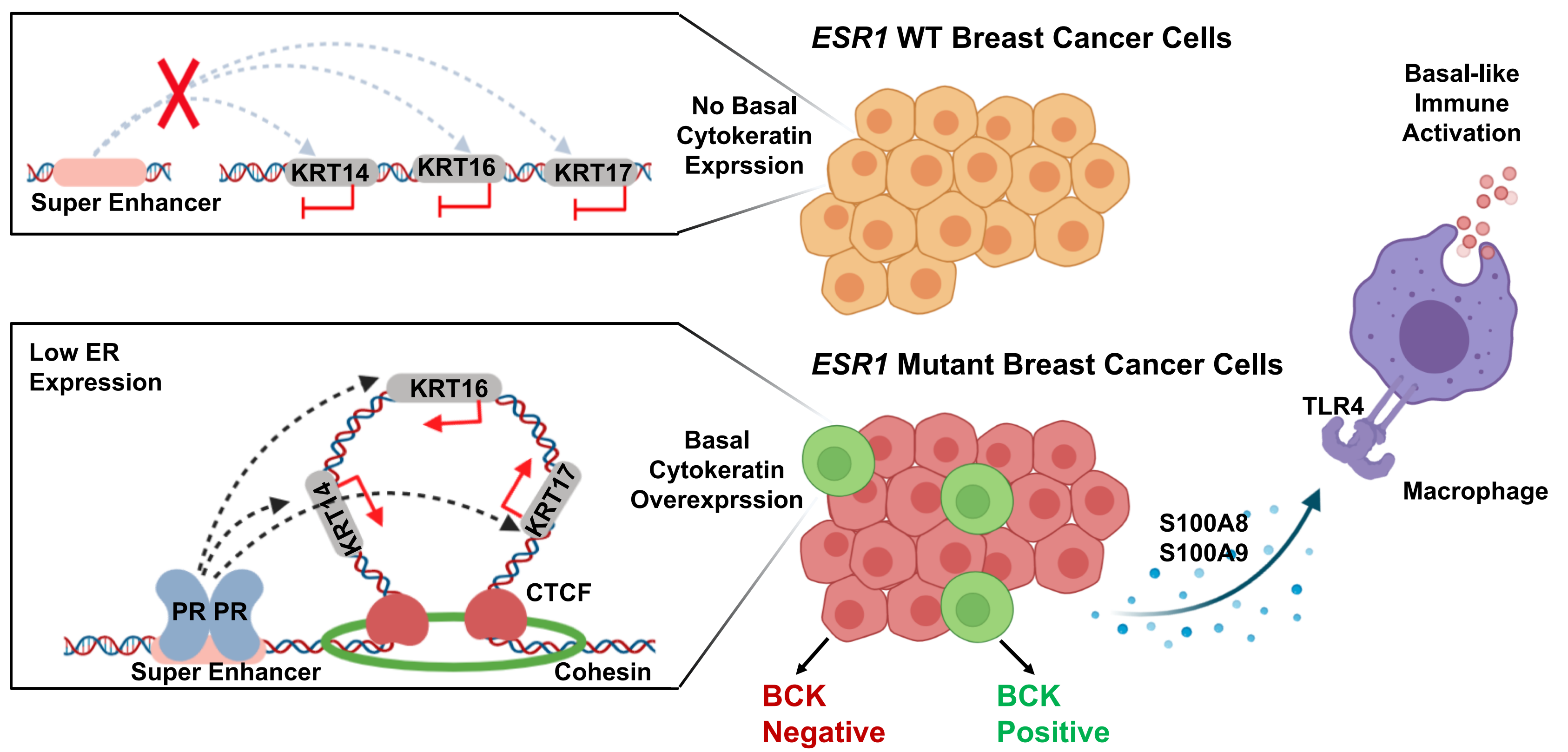


Figure 7. Schema of proposed mechanisms of basal cytokeratin induction in *ESR1* mutant breast cancer.