

1 **RIPK3 upregulation confers robust proliferation and collateral cystine-**  
2 **dependence on breast cancer recurrence**

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19 Running title: RIPK3 regulates YAP/TAZ-mediated cell proliferation.

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26 **Abstract**

27           The molecular and genetic basis of tumor recurrence is complex and poorly  
28 understood. RIPK3 is a key effector in programmed necrotic cell death and, therefore,  
29 its expression is frequently suppressed in primary tumors. In a transcriptome profiling  
30 between primary and recurrent breast tumor cells from a murine model of breast  
31 cancer recurrence, we found that RIPK3, while absent in primary tumor cells, is  
32 dramatically re-expressed in recurrent breast tumor cells by an epigenetic mechanism.  
33 Unexpectedly, we found that RIPK3 knockdown in recurrent tumor cells reduced  
34 clonogenic growth, causing cytokinesis failure, p53 stabilization, and repressed the  
35 activities of YAP/TAZ. These data uncover a surprising role of the pro-necroptotic  
36 RIPK3 kinase in enabling productive cell cycle during tumor recurrence. Remarkably,  
37 high RIPK3 expression also rendered recurrent tumor cells exquisitely dependent on  
38 extracellular cystine and undergo programmed necrosis upon cystine deprivation. The  
39 induction of RIPK3 in recurrent tumors unravels an unexpected mechanism that  
40 paradoxically confers on tumors both growth advantage and necrotic vulnerability,  
41 providing potential strategies to eradicate recurrent tumors.

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## 51 **Introduction**

52 While significant progress has been made for the diagnosis and treatment of  
53 primary tumors, the emergence of recurrent tumors after the initial response to  
54 treatments still poses significant clinical challenges. Recurrent breast tumors are  
55 generally incurable and unresponsive to the treatments effective for primary tumors <sup>1</sup>.  
56 Several factors have shown to be associated with breast tumor recurrence, including  
57 the age when primary tumor is diagnosed <sup>2, 3</sup>, lymph node status, tumor size,  
58 histological grade<sup>4, 5, 6</sup>, the status of estrogen receptor (ER), progesterone receptor  
59 (PR) and the expression of human epidermal growth factor receptor 2 (HER2)<sup>7, 8, 9</sup>.  
60 However, the molecular and genetic events that lead to tumor recurrence remain  
61 largely unknown.

62 To study the mechanism of tumor recurrence, genetically engineered mouse  
63 (GEM) models of recurrent breast cancers have been established. Utilizing the  
64 doxycycline-inducible system, the expression of specific oncogenes can be  
65 conditionally expressed and withdrawn in the mammary gland <sup>10, 11, 12, 13, 14</sup>. In the bi-  
66 transgenic mice expressing an MMTV-rtTA (MTB) and inducible Neu (homolog of  
67 HER2) oncogene (TetO-neu; TAN), mammary adenocarcinomas can be induced by  
68 the administration of doxycycline and regressed after doxycycline withdrawal <sup>10, 11, 12,</sup>  
69 <sup>13, 14</sup>. Importantly, the recurrent tumors will eventually emerge in most mice after the  
70 expression of the oncogene is turned off <sup>10, 11, 12, 13, 14</sup>. This tumor recurrent model  
71 bears significant similarities to human breast cancer recurrence in several important  
72 ways: (1) Tumor recurrence occurs over a long timeframe relative to the lifespan of  
73 the mouse, similar to the timing of recurrences in human breast cancer; (2) During the  
74 latency period before recurrent tumor formation, residual tumor cells remain in the  
75 mouse, analogous to minimal residual disease in patients; (3) The formation of

76 recurrent tumors is independent from the initial oncogene of the primary tumors,  
77 reminiscent of the finding that recurrent tumors from HER2-amplified breast cancers  
78 often lose HER2 amplification and become unresponsive to HER2 inhibition; (4)  
79 Recurrent breast cancer is often more aggressive than the initial primary tumor and  
80 resistant to therapies that were effective against the primary tumor. Breast cancer  
81 recurrence in human and GEM also share significant similarities in molecular  
82 pathways and clinical courses. For example, recurrent tumor cells of GEM typically  
83 acquired an epithelial-to-mesenchymal transition (EMT) phenotype, a hallmark of  
84 breast cancer recurrence <sup>13, 15</sup>. Unfortunately, while many signaling pathways are  
85 found to be enriched in “recurrent tumor”, most of these recurrent-enriched pathways  
86 are not readily amenable to therapeutic intervention. Therefore, there are still  
87 significant need for novel therapeutic approaches that target the recurrent tumor cells.

88         One relative unexplored aspect of recurrent tumor is the metabolic  
89 reprogramming and potential nutrient addiction. Previously, by systematic removal of  
90 individual amino acids we demonstrated that renal cell carcinomas and triple-negative  
91 breast cancer cells (TNBC) are highly susceptible to cystine deprivation or inhibitors  
92 of cystine/glutamate antiporter (xCT) that block the cystine import <sup>16, 17</sup>. Although  
93 cystine is not an essential amino acid, the imported cystine is broken down to cysteine,  
94 the limiting precursor of glutathione (GSH). GSH is a crucial antioxidant to decrease  
95 reactive oxygen species (ROS) in cells <sup>18</sup>. Therefore, depletion of cystine will result in  
96 the depletion of GSH, unopposed surge of ROS, which triggers programmed necrosis  
97 <sup>19</sup>. Cystine deprivation activates the Receptor Interacting Serine/Threonine Kinase 1  
98 (RIPK1), which recruits and promotes RIPK3 autophosphorylation. The activated  
99 RIPK3, in turn, leads to the phosphorylation and polymerization of Mixed Lineage  
100 Kinase Domain Like Pseudokinase (MLKL), resulting in the membrane rupture and

101 execution of necrosis<sup>20, 21</sup>. Accordingly, as a part of death-evading strategy, RIPK3  
102 expression is often silenced in primary tumors due to the promoter methylation<sup>22, 23</sup>.

103 Here we report that the RIPK3 is re-expressed in recurrent tumor cells and  
104 RIPK3 activity is required for productive proliferation of recurrent tumor cells. However,  
105 this exaggerated re-expression of RIPK3 also renders the recurrent tumor cells  
106 uniquely vulnerable to the necroptotic death triggered by cystine deprivation and xCT  
107 inhibitor treatment. Thus, RIPK3-dependent proliferation of recurrent tumor cells  
108 creates the collateral vulnerability to cystine deprivation that can serve as a strategy  
109 to exterminate recurrent tumor cells therapeutically.

110

## 111 **Results**

### 112 *Exaggerated expression of RIPK3 in the recurrent breast tumor cells*

113 To investigate the basis of phenotypic differences between primary and  
114 recurrent tumors, tumor cells were isolated and expanded from HER2 driven murine  
115 MTB/TAN model before the oncogenic withdrawal (primary tumors) and after the  
116 recurrence (recurrent tumors)<sup>12</sup>. To identify differentially expressed genes important  
117 for tumor recurrence, we have previously performed microarrays to compare the  
118 differences in the transcriptome landscape between primary and recurrent tumor cells  
119 (GEO: GSE116513)<sup>24</sup>. This comparison validates the previously reported upregulation  
120 of Ceramide Kinase (*Cerk*)<sup>25</sup> and downregulation of Prostate Apoptosis Response 4  
121 (*Par-4*)<sup>26</sup> in the recurrent tumor cells (Figure 1A). Also, recurrent tumor cells  
122 expressed a higher level of EMT-driving Snail Family Transcriptional Repressor 1  
123 (*Snai1*),<sup>13</sup>(Figure 1A), consistent with an enrichment of EMT by Gene Set Enrichment  
124 Analysis (GSEA) (Figure 1B). Therefore, these data confirm many distinct gene  
125 expression patterns reported between the primary and recurrent tumor cells.

126           When we examined the expression of genes involved in the programmed  
127 necrosis<sup>20</sup>, we noted a consistent and robust over-expression of *Ripk3* in the recurrent  
128 tumors cells (Figure 1A). RT-PCR validated the dramatically increased expression of  
129 *Ripk3* mRNA in the recurrent tumor cells (Figure 1C). In addition, Western blots  
130 revealed that RIPK3 protein, while almost entirely absent in the primary tumor cells,  
131 was abundantly expressed in the recurrent tumor cells (Figure 1D). In comparison,  
132 RIPK1 and MLKL proteins, the best recognized upstream regulator and downstream  
133 target of RIPK3, respectively, were found to be expressed at similar levels in the  
134 primary and recurrent tumor cells (Figure 1D). Similar *Ripk3* mRNA over-expression  
135 is also noted in a panel of mouse recurrent breast tumors, when compared with  
136 primary breast tumors (Figure 1E). The absence of RIPK3 protein expression in  
137 primary tumor cells was previously noted and assumed to be an evolutionary strategy  
138 of tumors to escapes programmed necrosis as part of the cancer hallmarks<sup>22, 27</sup>.  
139 Therefore, the absence of RIPK3 in primary tumor cells is consistent with these  
140 reports. However, the re-expression of RIPK3 in the recurrent tumor cells was  
141 unexpected.

142           This observation is supported by two human dataset of gene expression  
143 comparison between primary breast cancer and matching lymph node metastasis<sup>28</sup>.  
144<sup>29</sup>. First, *RIPK3* mRNA was significantly increased by 2.08-fold in metastatic tumors  
145 when compared with primary human tumors (Supplemental Table 1)<sup>28</sup>. Another human  
146 dataset (GSE61723)<sup>29</sup> that compared 16 pairs of primary breast cancer and matching  
147 lymph node metastasis, also showed an increase in *RIPK3* mRNA expression in 11  
148 out of 16 pairs with an overall significant upregulation (Figure 1F). Collectively, these  
149 data indicate the upregulation of RIPK3 expression occurs in recurrent tumors in both  
150 a mouse model and two human studies.

151

152 *Epigenetic regulation of Ripk3 in the primary vs. recurrent tumor cells*

153 To understand the basis of the *Ripk3* mRNA upregulation in the recurrent tumor  
154 cells, we investigated the epigenetic landscape of the regulatory regions of *Ripk3* gene  
155 in the primary and recurrent tumor cells by ChIP-sequencing. Consistent with the  
156 transcriptional upregulation in the recurrent tumor cells, RNA Polymerase II  
157 dramatically occupied the regulatory regions of *Ripk3* gene in the recurrent tumor cells,  
158 but not in the primary tumor cells (Figure 2A). Next, we compared the ChIP-seq data  
159 of activating epigenetic histone markers, H3K9Ac and H3K4me3, in the regulatory  
160 regions of *Ripk3*. We found that the regulatory regions of *Ripk3* gene adjacent to the  
161 RNA polymerase II binding site were highly enriched for these activating histone  
162 markers in the recurrent tumor cells, but not in the primary tumor cells (Figure 2A).

163 To further determine the epigenetic alterations of *Ripk3*, we designed two sets  
164 of primers that cover the promoters (-291 to -165), transcriptional start site (TSS, -84  
165 to +51) (Figure 2B) to measure the epigenetic changes by ChIP-PCR. We found that  
166 the promoter and TSS of *Ripk3* gene are marked by the activation markers (H3K4me3,  
167 H3K9Ac) and RNA polymerase II occupancy only in the recurrent tumor cells (Figure  
168 2B). Reciprocally, we found that these *Ripk3* regulatory regions are marked by the  
169 silencing markers (H3K27me3 and K3K9me2) in the primary tumor cells, but not in the  
170 recurrent tumor cells (Figure 2B).

171 We further performed bisulfite sequencing to measure the degree of DNA  
172 methylation of the CpG island in the *Ripk3* regulatory regions (-150 to +310) (Figure  
173 2C). We found that most of the cytosines in the *Ripk3* CpG Island are methylated in  
174 the primary tumor cells, but un-methylated in the recurrent tumor cells (Figure 2C).  
175 Together, these data indicate that epigenetic changes in the histone modification and

176 DNA methylations are likely responsible for the silencing of *Ripk3* in the primary tumor  
177 cells and robust expression in the recurrent tumor cells.

178

### 179 *Ripk3* knockdown triggers mitotic defects and p53 activation

180         Given the unexpected robust expression of RIPK3 in the recurrent tumors, we  
181 further investigated its functional role in recurrent tumor cells. First, we performed  
182 clonogenic assay to determine whether silencing *Ripk3* in primary or recurrent tumors  
183 affects their capacity to proliferate and form colonies (Figure 3A-B, Supplemental  
184 Figure 1A-B). We found that *Ripk3* knockdown by two independent shRNAs  
185 significantly reduced colony formation in the recurrent tumor cells (Figure 3A-B), but  
186 not in the primary cells (Supplemental Figure 1A-B). These data suggest that RIPK3  
187 is crucial for the proliferation and survival of recurrent tumor cells.

188         MLKL is the downstream effector of RIPK3 in the execution of programmed  
189 necrosis<sup>21</sup>. We found that *Mkl1* silencing in recurrent tumor cells recapitulated the  
190 effect of *Ripk3* knockdown and suppressed colony formation (Supplemental figure 1C-  
191 D). Consistent with these findings, treatment with an MLKL inhibitor, necrosulfonamide  
192 (NSA)<sup>21, 30</sup>, also reduced colony formation of recurrent tumor cells (Supplemental  
193 figure 1E-F). These data show that the canonical necrosis-driving RIPK3-MLKL  
194 signaling axis is required for cell proliferation and clonogenic growth in recurrent breast  
195 tumor cells.

196         To understand the mechanisms by which *Ripk3* knockdown the reduced  
197 clonogenic capacity of recurrent tumor cells, we investigated the transcriptional  
198 responses to *Ripk3* knockdown by RNAseq (submitted to GEO: GSE124634, reviewer  
199 token: wxklcoaixtgllsp) (Figure 3C). We found downregulation of several mitotic  
200 regulators in *Ripk3* knockdown cells, including Aurora B and *Mklp1* (Figure 3C), as



201 well as the depletion of the reactome to mitosis geneset by GSEA (Figure 3D).  
202 Therefore, we used fluorescence microscopy to investigate the potential impact of  
203 RIPK3 on mitosis. *Ripk3* knockdown dramatically increased the number of binucleated  
204 cells by ~20 folds (Figure 3E-F). These data suggest that *Ripk3* is involved in the  
205 proper execution of mitosis in the recurrent tumor cells. Binucleated/multinucleated  
206 cells generally result from cytokinesis failure<sup>31</sup>. Previous study showed that  
207 cytokinesis failure can lead to the activation of tumor suppressor *p53*<sup>32</sup>. By GSEA  
208 analysis, we found the enrichment of genes in *p53* signaling pathway (Figure 3G) and  
209 confirmed the upregulation of *p53* target genes, *Mdm2* and *p21*, in *Ripk3* knockdown  
210 cells (Figure 3C). Indeed, *p53* protein is phosphorylated at Ser15 upon *Ripk3* silencing  
211 (Figure 3H). Phosphorylation on Ser15 led to a weak interaction between *p53* and its  
212 negative regulator *Mdm2*<sup>33</sup>, which in terms stabilize *p53* accumulation (Figure 3H).  
213 Collectively, these data suggest that robust *Ripk3* expression in recurrent tumor cells  
214 is critical for the proper mitotic progression and cell proliferation.

215         Given that *Ripk3* knockdown increased binucleated cells, which cause genomic  
216 instability<sup>34</sup>, we speculated that *Ripk3* knockdown may lead to aneuploidy. A recent  
217 report has made the scores of aneuploidy available in a pan-cancer TCGA dataset<sup>35</sup>.  
218 Therefore, we correlated the level of *RIPK3* expression with its aneuploidy score in  
219 breast cancer patients (Figure 3I). Our results indicate that low levels of *RIPK3* mRNA  
220 expression is significantly associated with higher aneuploidy in breast cancers (Figure  
221 3I). These data in human breast tumors further support the concept that *RIPK3* is  
222 critical in preventing chromosome instability and aneuploidy in recurrent tumor cells.

223

224 *Ripk3* knockdown represses YAP/TAZ pathways

225 Cytokinesis failure can activate Hippo tumor suppressor pathway<sup>32</sup> and  
226 inactivate the two Hippo pathway effectors, YAP (Yes Associated Protein 1 encoded  
227 by *YAP1*) and TAZ (transcriptional coactivator with PDZ-binding motif, encoded by  
228 *WWTR1*). These proteins are coactivators of TEAD family transcription factors  
229 mediating the expressions of proliferative and oncogenesis genes<sup>36</sup>. When Hippo is  
230 on, YAP/TAZ is inactivated by phosphorylation and exclusion from the nucleus. When  
231 Hippo is off, YAP/TAZ is localized in the nucleus and able to interact with TEAD and  
232 leads to downstream gene expression<sup>36</sup>. We found that *Ripk3* silencing in recurrent  
233 tumor cells led to a depletion of YAP/TAZ signature by GSEA (Figure 4A). RT-PCR  
234 confirmed that two canonical YAP/TAZ target genes: *Ctgf* and *Cyr61*, were  
235 dramatically repressed upon *Ripk3* knockdown (Figure 3C and Figure 4B). In addition,  
236 we examined how *Ripk3* silencing affects the sub-cellular localization of YAP and TAZ  
237 by nuclear/cytosol fractionation (Figure 4C). While *Ripk3* silencing slightly reduced the  
238 level of nuclear YAP, it significantly depleted nuclear TAZ (to ~18%) with a  
239 corresponding increase in the cytosolic TAZ (Figure 4C). Confocal microscopy further  
240 confirmed the reduced nuclear YAP/TAZ upon *Ripk3* silencing (Figure 4D). Thus, we  
241 speculated that the depletion of YAP/TAZ in the nucleus under *Ripk3* silencing may  
242 contribute to low efficiency of colony formation and cell proliferation. To test this  
243 hypothesis, we further over-expressed constitutively active mutants of YAP/TAZ, YAP  
244 S127A and TAZ S89A<sup>37, 38</sup> in *Ripk3* knockdown cells (Figure 4E-F). We observed a  
245 complete rescue by TAZ S89A expression under *Ripk3* knockdown whereas YAP  
246 S127A partially rescued colony formation (Figure 4E-F). These data suggest that  
247 reduced nuclear YAP/TAZ levels and activities, especially TAZ, contribute significantly

248 to the defect of clonogenic formation induced by *Ripk3* silencing in recurrent tumor  
249 cells.

250

251 *Recurrent breast tumor cells are uniquely addicted to exogenous cystine*

252         Recent studies have indicated that therapy-resistant and mesenchymal tumor  
253 cells, two features seen in the recurrent tumor cells, become more sensitive to cell  
254 death induced by cystine deprivation<sup>17, 39</sup>. Cystine is imported into mammalian cells  
255 in exchange of the export of glutamate via the xCT transporter<sup>40</sup>, which can be blocked  
256 by xCT inhibitors, such as the erastin or sulfasalazine<sup>41, 42, 43</sup>. We have found that the  
257 cystine deprivation can trigger extensive cell death in renal cell carcinomas<sup>16</sup> and  
258 triple negative breast cancer cells<sup>17</sup>. Given the unexpected robust level of RIPK3  
259 expression in recurrent breast cancer cells, we investigated whether recurrent breast  
260 tumor cells are particularly vulnerable to cell death triggered by cystine deprivation or  
261 erastin. We subjected two primary and two recurrent tumor cell lines to normal (200  
262  $\mu$ M cystine) or cystine-deprived (2.5  $\mu$ M of cystine) media for 16 hours and determined  
263 the cell viability using crystal violet. We found that cystine deprivation eliminated most  
264 of recurrent tumor cells, but only had modest effects on primary tumor cells (Figure  
265 5A). Under varying degrees of cystine deprivation, the recurrent tumor cells were  
266 largely eliminated under 5 $\mu$ M of cystine (Figure 5B). In contrast, the primary tumor  
267 cells still maintained ~50% viability even at 0.625  $\mu$ M of cystine (Figure 5B).  
268 Collectively, these cell viability assays consistently showed that recurrent tumor cells  
269 are much more sensitive to cystine deprivation.

270         Alternatively, we examined whether primary and recurrent breast tumor cells  
271 have different sensitivity to erastin, a potent xCT inhibitor. Consistently, we found  
272 recurrent tumor cells, when compared with primary tumor cells, were more sensitive

273 to erastin-induced cell death examined by crystal violet staining (Figure 5C) and  
274 CellTiter-Glo assay (Figure 5D). While recurrent tumor cells were largely eliminated  
275 between 0.5~1  $\mu$ M erastin, the primary cells survived more than 8  $\mu$ M of erastin with  
276 ~75% viability (Figure 5D). Such recurrent-specific erastin sensitivities are further  
277 confirmed by the higher levels of protease release in the recurrent tumor cells an  
278 indication of cell membrane breakage and death (Figure 5E).

279 Thus, we further analyzed the protein expression of RIPK3 and MLKL in primary  
280 and recurrent tumor cell lines after erastin treatment. We found that RIPK3 protein is  
281 only expressed in recurrent tumor cells and modestly elevated by erastin treatment  
282 (Supplemental Figure 2A). An upregulated base level of phosphorylated MLKL is  
283 noted in recurrent tumor cells when comparing to primary tumor cells (Supplemental  
284 Figure 2A). Moreover, silencing of *Ripk3* abolished the MLKL phosphorylation  
285 (Supplemental Figure 2B). Therefore, the elevated RIPK3 proteins and constitutive  
286 MLKL phosphorylation may prime the recurrent tumor cells to cell death triggered by  
287 erastin or cystine deprivation.

288 Erastin is considered to trigger to cell death by ferroptosis, a programmed cell  
289 death distinct from apoptosis and programmed necrosis <sup>42</sup>. However, at low dose of  
290 erastin, we have previously found that necrosis pathway and RIPK3 is also required  
291 for programmed cell death <sup>16</sup>. Given the elevated RIPK3 in recurrent tumor cells, we  
292 further determined the cell death mechanisms. In addition to low dose of erastin, we  
293 used different inhibitors to define the cell death mechanisms caused by erastin. We  
294 found that the apoptosis inhibitor Z-Vad did not rescue the erastin-induced death. In  
295 contrast, both ferroptosis inhibitor (ferrostatin-1) <sup>44</sup> and necrosis inhibitor (necrostatin-  
296 5) <sup>45</sup> robustly rescued the cell death, suggesting the potential role of RIPK3 in the cell  
297 death triggered by erastin (Figure 5F). While the requirement for RIPK3 may not be

298 generally applicable to all erastin-induced cell death, RIPK3 may be particularly critical  
299 in the recurrent tumor cells with high RIPK3 expression and constitutive MLKL  
300 phosphorylation.

301

302 *Ripk3 over-expression contribute to the recurrent-specific cystine addiction*

303 To examine whether high *Ripk3* expression in recurrent tumor cells contributes  
304 to its vulnerability to cystine deprivation, we knocked down *Ripk3* by two independent  
305 shRNAs and found a significant reduction of erastin-induced cell death with ~70-80%  
306 of viability (Figure 6A), while erastin (1  $\mu$ M) eliminated the control recurrent tumor cells  
307 to less than 10% cell viability (Figure 6A). Similar results were also obtained by crystal  
308 violet staining and follow-up quantification (Figure 6B-C). Therefore, the robust *Ripk3*  
309 expression contributes to the cystine addiction phenotypes of recurrent tumor cells.

310 Since the MLKL phosphorylation by RIPK3 is required for MLKL oligomerization  
311 and activation of necrosis, we further inhibited MLKL oligomerization by NSA<sup>21,30</sup>. We  
312 found NSA was able to rescue the erastin-induced cell death of recurrent tumor cells  
313 using either protease release assay (Figure 6D) or CellTiter-Glo assay (Figure 6E).  
314 Therefore, the exaggerated RIPK3 expression and MLKL phosphorylation, not only  
315 enable recurrent tumor cells to proliferate, but also contribute to their sensitivity to  
316 cystine deprivation and erastin treatment. These results may be limited to the recurrent  
317 tumor cells with high RIPK3 expression and constitutive MLKL phosphorylation that  
318 pose the cells to necrosis-inducing signaling triggered by cystine deprivation or erastin.

319

## 320 **Discussion**

321 Global changes in epigenetic landscapes are a hallmark of cancer. While  
322 RIPK3 expression is found in most of the normal tissue, the promoter region of *RIPK3*

323 usually becomes highly methylated in cancer cells leading to absence of *RIPK3*  
324 expression<sup>22, 23, 27</sup>. Since *RIPK3* determines necrosis by phosphorylating *MLKL*<sup>30</sup>,  
325 absence of *RIPK3* expression can be considered as adaptation process for tumor cells  
326 to evade death from various necrosis-triggering signals. In this study, primary tumor  
327 cells indeed showed low *Ripk3* expression (Figure 1C-F). However, after withdrawing  
328 of oncogene, the recurrent tumor cell lines showed exaggerating amount of *RIPK3*  
329 that triggers constitutive *MLKL* phosphorylation (Figure 1C-F and Supplemental Figure  
330 2A); however, the activation of the *RIPK3-MLKL* complex does not lead to  
331 programmed necrosis (Supplemental Figure 2A). Both *RIPK3* and *MLKL* silencing  
332 rendered recurrent tumor cells inefficient in colony formation (Figure 3A-B and  
333 Supplemental Figure 1C-D), which support the potential role of *RIPK3-MLKL* axis in  
334 supporting cell growth. The upregulation of *RIPK3*, therefore, offers selective  
335 advantages for the recurrent tumor cells.

336 Although the detailed mechanism remains to be discovered, our data revealed  
337 that *Ripk3* silencing increased cytokinesis failure, p53 stabilization and inactivation of  
338 *YAP/TAZ* signaling pathway<sup>32</sup>. Thus, exaggerated expression of *RIPK3* contribute to  
339 the proliferation of the recurrent cells through *YAP/TAZ* activities. Consistently, *RIPK3*  
340 has been shown to confer survival and adaptive advantages in selected tumor  
341 settings. Knockdown of *RIPK3* in MDA-MB-231 breast cancer cells contribute to arrest  
342 in *in vivo* tumor growth<sup>46</sup>. Similarly, the *RIPK1/RIPK3* are highly expressed in  
343 pancreatic cancers and the *in vivo* deletion of these necrosis proteins delayed  
344 oncogenic progression<sup>47</sup>.

345 Our studies also have significant therapeutic implications. Erastin can induce a  
346 non-apoptotic form of cell death named ferroptosis<sup>42</sup>. Although ferroptosis is  
347 considered to be regulated by glutathione peroxidase 4 and independent of

348 RIP1/RIPK3/MLKL-mediated necrosis<sup>48</sup>, our previous study has shown that low dose  
349 of erastin can induce necrosis-mediated cell death<sup>16</sup>, which is supported by another  
350 study<sup>49</sup>. Consistent with our data, in the recurrent tumor cells with exaggerated RIPK3  
351 expression, cell death induced by erastin can be mitigated by knockdown of RIPK3  
352 (Figure 6). Furthermore, both Nec-5 and ferrostatin-1 rescued erastin induced cell  
353 death (Figure 5F). These data indicate that high RIPK3 expression may contribute to  
354 its sensitivities to cell death induced by cystine deprivation. While tumor recurrence is  
355 usually considered incurable, this finding suggests that the collateral vulnerability to  
356 RIPK3 mediated necrosis may hold therapeutic potential. *In vivo* cystine removal  
357 using recombinant cyst(e)inase<sup>50</sup> and inhibitors of cystine importer xCT<sup>51</sup> are being  
358 developed for clinical translation. Our data suggest that the recurrent tumors  
359 expressing high level of the necrosis components may be uniquely sensitive to these  
360 therapeutic approaches.

361

## 362 **Methods**

### 363 *Cell culture*

364 Primary and recurrent MTB/TAN tumor cells described previously<sup>13</sup> were  
365 cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-11995)  
366 supplemented with 10% fetal bovine serum and 1 × antibiotics (penicillin, 10,000 UI/ml  
367 and streptomycin, 10,000 UI/ml). For primary cells, 10 ng/ml EGF, 5 µg/ml insulin, 1  
368 µg/ml hydrocortisone, 5 µg/ml prolactin, 1 µM progesterone and 2 µg/ml doxycycline  
369 were added to the media to maintain HER2/neu expression. For recurrent cells, 10  
370 ng/ml EGF and 5 µg/ml insulin were added to the media. Both primary and recurrent  
371 cell lines were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

### 372 *ShRNA and lentivirus infections*

373 RIPK3 shRNA targeting mouse RIPK3 RNA were purchase from Sigma  
374 (TRCN0000022536, TRCN0000424625). MLKL shRNA targeting mouse MLKL RNA  
375 were purchase from Sigma (TRCN0000022599, TRCN000022602).Lentivirus  
376 expressing RIPK3 shRNA was generated by transfecting HEK-293T cells in 6 well  
377 plate with a 1: 0.1: 1 ratio of pMDG2: pVSVG: pLKO.1 with TransIT-LT1 transfection  
378 reagent (Mirus). After filtering through 0.45 µm of cellulose acetate membrane (VWR,  
379 28145-481), lentivirus (250 ul) were added to a 60mm dish of recurrent cells with  
380 polybrene (8ug/ml). After 24 hours of incubation, recurrent cells were further selected  
381 with puromycin (5 µg/ml) to increase knockdown efficiency.

#### 382 *Cell viability and cytotoxicity*

383 Cell viability assay was performed by using CellTiter-Glo luminescent cell  
384 viability assay (Promega) following manufacturer's protocol. Cytotoxicity was  
385 determined by membrane rupture and protease release using CellTox Green  
386 cytotoxicity assay (Promega) following manufacturer's protocol.

#### 387 *Western blots*

388 Primary and recurrent tumor cell lines were harvested and washed once with  
389 ice cold PBS. The samples were then resuspended in NP-40 buffer with protease and  
390 phosphatase inhibitors and lysed by incubating in at 4°C with constant vortex for 30min,  
391 then spun down at 13000 rpm for 10 min at 4°C. Supernatant was transferred to  
392 another tube, and protein concentration was measured by BCA protein assay kit  
393 (#23225, ThermoFisher). Western blotting was performed as previously described<sup>52</sup>.  
394 Nuclear and cytoplasmic extraction for YAP/TAZ was performed by following  
395 manufacturer's protocol (#78835, ThermoFisher). Quantification of YAP/TAZ was  
396 performed by Image J software and normalized to Lamin A/C protein level. Around 20  
397 ug of protein was loaded on 8% SDS-PAGE gels, transferred to PDVF membrane,



398 blocked with 5% non-fat milk in 1xTBST, incubated with primary antibodies overnight  
399 at 4°C. Primary antibodies: RIPK1 (1:1000, 610458, BD biosciences); RIPK3 (1:1000,  
400 sc-374639, Santa Cruz); GAPDH (1:2000, sc-25778, Santa Cruz); Phospho-MLKL  
401 (Ser345) (1:1000, #62233, Cell signaling); MLKL (1:1000, #28640, Cell  
402 signaling); Pho-p53-S15 (1:1000, #92845, Cell signaling); Lamin A/C  
403 (1:1000, #4777T, Cell signaling); TAZ (1:1000, 560235, BD biosciences); YAP  
404 (1:1000, sc376830, Santa Cruz)

#### 405 *Quantitative real-time PCR*

406 RNA from the samples was extracted by the RNeasy Mini Kit (Qiagen) following  
407 the manufacturer's protocol. RNA was reverse transcribed to cDNA by random  
408 hexamers and SuperScript II (Invitrogen). Quantitative real-time PCR was performed  
409 following the manufacturer's protocol by using Power SYBR Green PCR Mix (Applied  
410 Biosystems) and StepOnePlus Real-time PCR system (Applied Biosystems). Samples  
411 were biologically triplicated for mean $\pm$  SEM. Data were representative of three  
412 independent repeats. Mouse beta-actin (reference gene) primers: sense, 5'- GGC  
413 TGT ATT CCC CTC CAT CG -3', antisense, 5'- CCA GTT GGT AAC AAT GCC ATG  
414 T-3'; Mouse RIPK3 primers: sense, 5'- TCT GTC AAG TTA TGG CCT ACT GG-3',  
415 antisense, 5'-GGA ACA CGA CTC CGA ACC C-3'. Mouse CTGF primers: sense, 5'-  
416 GCC TAC CGA CTG GAA GAC AC-3', antisense, 5'- GGA TGC ACT TTT TGC CCT  
417 TCT TA-3'. Mouse CYR61 primers: sense, 5'- CTG CGC TAA ACA ACT CAA CGA-  
418 3', antisense, 5'- GCA GAT CCC TTT CAG AGC GG-3'.

#### 419 *ChIP-Seq and ChIP-PCR*

420 ChIP-Seq and ChIP-PCR were performed as described previously<sup>24</sup>. Tumor  
421 cells were crosslinked in 1% formaldehyde (Sigma) for 10 minutes, prior to quenching  
422 with 250 mM glycine. DNA was sonicated to an average shear length of ~250-450 bp  
423 length. Lysates were precleared with protein A/G beads and immunoprecipitated with  
424 5 µg of H3K9ac, H3K4me3, H3K27me3, H3K9me2, and RNAPol2 antibodies  
425 purchased from Abcam. DNA was sequentially washed with wash buffers. DNA was  
426 eluted from washed beads and reverse cross-linked with concentrated NaCl overnight.  
427 After reverse cross-linking, proteins were digested with Proteinase K and chelated with  
428 EDTA. DNA was purified using PCR purification columns (Qiagen) according to  
429 manufacturer instructions. All qPCR reactions were carried out with SYBR green (Bio-  
430 Rad) Primers for promoter region of RIPK3: sense, 5'- CTT GGA CCC CTT AGC TCC  
431 AC-3', antisense, 5'-GTA CCT GGC CCA AGA CAA CC-3'. Primers for TSS region of  
432 RIPK3: sense, 5'- CCC GGA CTT TGA ATG AGC GA-3', antisense, 5'-CTC GGG  
433 TGG AAG CAG TTT CA-3'. Ct values were normalized to input DNA.

434 Immunoprecipitated DNA was sequenced on Illumina HiSeq 4000 sequencer  
435 with 50 bp single reads at an approximate depth of 55 million reads per sample.  
436 Sequencing reads underwent strict quality control processing with the TrimGalore  
437 package and were mapped to the mm10 genome using Bowtie aligner. Alignment files  
438 were converted to bigwig files by binning reads into 100bp segments. H3K4me3,  
439 H3K9ac and RNAPol II tracks were visualized for the Ripk3 promoter by IGV desktop  
440 viewer (Broad Institute).

441

442 *Bisulfite sequencing*

443 Bisulfite sequencing was performed using the EpiTect Bisulfite Kit (Qiagen) according  
444 to manufacturer instructions. The Ripk3 promoter region was PCR amplified using  
445 primers designed following previous study<sup>53</sup>. Sense, 5'- AGA GAA TTC GGA TCC  
446 TGG AGT TAA GGG GTT TAA GAG AGA T-3', antisense, 5'-CTT CCA TGG CTC  
447 GAG CTT TAT CCC CTA CCT CAA AAA AAA C-3'. Amplified DNA was gel purified  
448 and transformed into competent bacteria. Ten independent bacterial colonies were  
449 sequenced for Ripk3, and DNA sequences were aligned with DNASTAR MegAlign  
450 software.

#### 451 *Immunofluorescence microscopy*

452 Recurrent tumor cells were washed once with PBS and fixed in 4%  
453 paraformaldehyde for 15 min, followed by permeabilization and blocking with 0.2%  
454 Triton X-100 and 2% BSA for 15 min. Primary antibodies were incubated with the cells  
455 for 1 hour. Immunofluorescence microscopy were performed using EVOS FL cell  
456 imaging system (ThermoFisher) or confocal microscope (880, Zeiss). Antibody: Alexa  
457 Fluor 594 Phalloidin (1:100, A12381, ThermoFisher); TAZ (1:100, 560235, BD  
458 biosciences); pho-histone H2AX-S139 (1:100, GTX628996, GeneTex).

#### 459 *RNA-seq and GESA*

460 TrimGalore toolkit is used to process RNA-seq data. It employs Cutadapt to trim  
461 low-quality bases and Illumina sequencing adapters from the 3' end of the reads.  
462 Reads (>20nt) after trimming were kept for further analysis. By using the STAR RNA-  
463 seq alignment tool, reads were mapped to the GRCm38v73 version of the mouse  
464 genome and transcriptome. If reads were mapped to a single genomic location, it were  
465 kept for subsequent analysis. Quantification of read counts of genes were performed  
466 using HTSeq. Only genes that had more than 10 reads in any given library were

467 further analyzed. DESeq2 Bioconductor package with the R statistical programming  
468 environment were applied for differential analysis to compare recurrent tumor cells  
469 with control or shRIPK3 silencing. The false discovery rate was calculated to control  
470 for multiple hypothesis testing. Gene set enrichment analysis was performed to  
471 identify gene ontology terms and pathways associated with altered gene expression  
472 for the comparisons between control and recurrent cells with shRIPK3 silencing.

### 473 *Statistical analysis*

474 Data represent the mean +/- the standard error of the mean. P-values were  
475 determined by two ANOVA test with Bonferroni post hoc tests or a two-tailed Student's  
476 t-test in Graphpad. Error bars represent SEM, and significance between samples is  
477 denoted as \*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.001.

### 478 *Data availability*

479 RNAseq for recurrent cells with shRIPK3 silencing has been deposited in the  
480 NCBI Genome Expression Omnibus (GEO, GSE124634). All data and reagents  
481 supporting the findings of this study are available from the authors upon reasonable  
482 request.

483

### 484 **Author contributions**

485 C.C.L. and J.T.C. conceived the experiments and wrote the manuscript. C.C.L.  
486 performed the majority of the experiments. J.T.C., T.P.Y. and J.A. supervised the  
487 work. N.M., Y.T.L., W.H.Y., X.T., L.H. and T.S. collaborated in the discussion and  
488 experiments. J.T.C., T.P.Y. and J.A. provided critical feedback.

489

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495

#### 496 **Conflict of interest statement**

497 The authors have declared that no conflict of interest exists.

498

#### 499 **Figure legends**

#### 500 **Figure 1 Transcriptome profiling of primary and recurrent tumor cells revealed** 501 **RIPK3 upregulation in recurrent cells**

502 (A) Heatmap of the transcriptional difference between two primary and two recurrent  
503 cell lines. Color scale indicates log<sub>2</sub>-fold-change. (B) GSEA analysis showed the  
504 enrichment of EMT geneset in the recurrent tumor cells. (C) *Ripk3* was highly  
505 expressed in recurrent tumor cells by RT-PCR. (D) Western blot showed a robust  
506 RIPK3 protein expression only in recurrent tumor cell lines. (E) Comparison of *Ripk3*  
507 RNA expression between 10 primary and 10 recurrent mouse tumors showed an  
508 overall increase in recurrent tumors. (F) Comparison of *RIPK3* expression between  
509 primary breast cancer and matching lymph node metastasis in human dataset  
510 (GSE61723). Bars show standard error of the mean. \* $p < 0.05$ , \*\*\* $p < 0.001$ , two-tailed  
511 Student's *t*-test.

#### 512 **Figure 2 Epigenetic landscape of the regulatory regions of *Ripk3* in the primary** 513 **vs. recurrent tumor cells**

514 (A) ChIP-Seq data showed the occupancy of RNA Pol II, H3K4me<sub>3</sub> and H3K9ac in  
515 regulatory regions of *Ripk3* of recurrent tumor cells (B) ChIP-qPCR analysis of

516 H3K4me3, H3K9ac, RNA pol II, H3K27me3, H3K9me2 enrichment at two indicated  
517 regions in the promoter of the *Ripk3* genes in two primary and two recurrent tumor cell  
518 lines. Data are presented as the percentage of input DNA. (C) The cytosine  
519 methylation of CpG dinucleotides (circles) within the *Ripk3* promoter and gene body (-  
520 150 to +310) for two primary and two recurrent tumor cell lines. Bisulfite-treated DNA  
521 was transformed into bacteria and 10 replicate colonies were sequenced (rows). Open  
522 circles denote unmethylated CpG dinucleotides, while closed circles denoted  
523 methylated CpG dinucleotides.

### 524 **Figure 3 *Ripk3* knockdown triggers p53 signaling and mitotic defects**

525 (A) *Ripk3* silencing decreased colony formation. Clonogenic assay was performed by  
526 plating 500 recurrent tumor cells to 6 well plates. After 10 days of incubation, cells  
527 were fixed with paraformaldehyde (4%) and stained with crystal violet. (B)  
528 Quantification of number of colony formation. (C) Heatmap of the transcriptional  
529 response to *Ripk3* silencing in recurrent cells with several affected genes indicated.  
530 (D) GSEA analysis showed depletion of Reactome Cell Cycle Mitosis upon *Ripk3*  
531 silencing. (E) *Ripk3* silencing dramatically increased binucleated cells. Recurrent cells  
532 were stained with DAPI (nucleus) and Alexa Flour 594 Phalloidin (F-actin). Scale bar,  
533 5 $\mu$ m. (F) Quantification of binucleated cells under *Ripk3* silencing. (G) GSEA analysis  
534 showed that *Ripk3* silencing enriched p53 signaling pathway. (H) *Ripk3* silencing led  
535 to the accumulation of p53 and increased Serine 15 phosphorylation. (I) Lower *RIPK3*  
536 expression in human breast cancers is associated with increased amount of  
537 aneuploidy. \* $p < 0.05$  ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , two-tailed Student's *t*-test. (B)  $n=4$  and  
538 (F)  $n=3$  independent repeats. Bars show standard error of the mean. (I)  $n=1024$ .

### 539 **Figure 4 *Ripk3* knockdown abolishes YAP/TAZ-dependent cell growth**

540 (A) GSEA analysis showed the depletion of YAP/TAZ transcriptional target geneset  
541 upon *Ripk3* silencing in recurrent cells. (B) RT-PCR validated the downregulation of  
542 *Ctgf* and *Cyr61*, two canonical YAP/TAZ target genes upon *Ripk3* knockdown. (C)  
543 Nuclear/cytosol fractionation showed the depletion of TAZ upon RIPK3 knockdown.  $\alpha$ -  
544 tubulin: cytosolic marker; Lamin A/C: nuclear marker. Relative YAP/TAZ ratio was  
545 determined by normalizing YAP/TAZ intensity to Lamin A/C using ImageJ. (D)  
546 Confocal microscopy confirmed the depletion of YAP/TAZ upon RIPK3 knockdown.  
547 Scale bar, 10 $\mu$ m. (E) Overexpression of constitutively active YAP S127A and TAZ  
548 S89A rescued the low colony formation upon *Ripk3* knockdown as quantified in (F).  
549 \* $p < 0.05$  ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , two-tailed Student's *t*-test.  $n=3$  independent repeats.  
550 Bars show standard error of the mean.

551 **Figure 5 Recurrent tumor cells are more sensitive to cystine deprivation and**  
552 **erastin-induced death**

553 (A) Recurrent tumor cells, when compared with primary tumor cells, were more  
554 sensitive to cystine deprivation. Two primary and two recurrent cell lines were  
555 incubated in full media (200  $\mu$ M) or cystine-deprived media (2.5  $\mu$ M) for 16 hours. The  
556 cells were then fixed with paraformaldehyde (4%) for crystal violet staining. (B)  
557 Recurrent tumor cells, when compared with primary tumor cells, were more sensitive  
558 to cell death under cystine deprivation. Primary and recurrent cells were incubated  
559 with decreasing level of cystine for 16 hours. The viability was then measured by ATP  
560 level using Celltiter Glo assay. (C) Recurrent tumor cells are more sensitive to erastin  
561 treatment than primary tumor cells. Two primary and two recurrent cell lines were  
562 incubated in erastin (1  $\mu$ M) or DMSO for 18 hours. The cells were then fixed for crystal  
563 violet staining. (D) Primary and recurrent tumor cells were treated with increasing  
564 indicated doses of erastin for 18 hours and the viability was measured by Celltiter Glo

565 assay. **(E)** Erastin induced more cell rupture and protease release in recurrent cells.  
566 Primary and recurrent cells were treated with 1 $\mu$ M of erastin for 16 hours. The media  
567 was then harvested for protease measurement. **(F)** Erastin-induced cell death was  
568 rescued by Nec-5 and Ferrostatin-1. Erastin (2  $\mu$ M) were treated at the same time with  
569 DMSO, Z-vad (20 $\mu$ M), Nec-5 (5  $\mu$ M) and Ferrostatin-1 (1  $\mu$ M) in recurrent cell lines for  
570 18 hours. The cell viability was then determined by Celltiter Glo assay. **(B,D)**  
571  $p < 0.0001$ , Two-way ANOVA,  $*p < 0.05$ ,  $***p < 0.001$ . Bonferroni post hoc tests. **(E,F)**  
572  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ , two-tailed Student's *t*-test.  $n = 3$  independent repeats.  
573 Bars show standard error of the mean.

574 **Figure 6 *Ripk3* over-expression contribute to the recurrent-specific cystine**  
575 **addiction**

576 **(A)** *Ripk3* knockdown mitigated the erastin-induced cell death. Recurrent cells  
577 transduced with control or two *Ripk3* shRNAs were treated with increasing dose of  
578 erastin for 16 hours. Cell viability was then measured by Celltiter Glo assay. **(B-C)**  
579 Recurrent cells transduced with control or two *Ripk3* shRNAs were treated with 0.5  
580  $\mu$ M of erastin for 16 hours before assessing their viability by crystal violet staining **(b)**,  
581 as quantified in **(c)**. **(D-E)** MLKL phosphorylation by RIPK3 contributed to the erastin-  
582 induced cell death. Inhibiting MLKL by compound inhibitor (NSA, 5  $\mu$ M) protected  
583 recurrent tumor cells from cell death under erastin treatment (0.5  $\mu$ M) when measured  
584 by protease release **(D)** or Celltiter Glo assay **(E)**. **(A,E)**  $p < 0.0001$ , Two-way ANOVA,  
585  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , Bonferroni post hoc tests. **(C,D)**  $*p < 0.05$ ;  $**p < 0.01$ ;  
586  $***p < 0.001$ , two-tailed Student's *t*-test.  $n = 3$  independent repeats. Bars show standard  
587 error of the mean.

588 **Supplemental Figure 1 *Mkl1* inhibition by genetic or chemical means decreases**  
589 **colony formation in recurrent cells**



590 (A) *Ripk3* knockdown did not alter colony formation in primary tumor cells. (B)  
591 Quantification of number of colony formation in (A). (C) *Mkl1* knockdown recapitulates  
592 the reduced clonogenic phenotype of *Ripk3* silencing in recurrent cells. (D)  
593 Quantification of number of colony formation in (C). (E) MLKL inhibitor (NSA, 5  $\mu$ M)  
594 recapitulates the reduced clonogenic phenotype of *Ripk3* silencing. (F) Quantification  
595 of number of colony formation in (E). N.S. not significant; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , two-  
596 tailed Student's *t*-test.  $n = 3$  independent repeats. Bars show standard error of the  
597 mean.

598 **Supplemental Figure 2 High RIPK3 expression in recurrent tumor cells is**  
599 **associated with the base-line phosphorylation of MLKL**

600 (A) The recurrent tumor cells with high RIPK3 protein expression have higher baseline  
601 MLKL phosphorylation. Primary and recurrent cell lines were treated with 2  $\mu$ M of  
602 erastin for 12 hours and lysed for Western blot with indicated antibodies. (B) *Ripk3*  
603 silencing abolished MLKL phosphorylation in recurrent tumor cells. Recurrent cell lines  
604 were transduced with control vector or two *Ripk3* shRNAs for 72 hours. The cells were  
605 then lysed for Western blot to measure indicated proteins.

606 **Supplemental Table 1 RIPK3 expression is elevated in metastatic tumors in**  
607 **human dataset**

608 RIPK3 expression in primary and metastatic tumors were evaluated by *in situ*  
609 hybridization on tissue arrays.

610

611

612 **References**

- 613 1. Kimbung S, Loman N, Hedenfalk I. Clinical and molecular complexity of  
614 breast cancer metastases. *Semin Cancer Biol* 2015, **35**: 85-95.

615

- 616 2. Kollias J, Elston CW, Ellis IO, Robertson JF, Blamey RW. Early-onset breast  
617 cancer--histopathological and prognostic considerations. *Br J Cancer* 1997,  
618 **75**(9): 1318-1323.  
619
- 620 3. Albain KS, Allred DC, Clark GM. Breast cancer outcome and predictors of  
621 outcome: are there age differentials? *J Natl Cancer Inst Monogr* 1994(16): 35-  
622 42.  
623
- 624 4. Chiang AC, Massague J. Molecular basis of metastasis. *N Engl J Med* 2008,  
625 **359**(26): 2814-2823.  
626
- 627 5. Carter CL, Allen C, Henson DE. Relation of tumor size, lymph node status,  
628 and survival in 24,740 breast cancer cases. *Cancer* 1989, **63**(1): 181-187.  
629
- 630 6. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The  
631 value of histological grade in breast cancer: experience from a large study  
632 with long-term follow-up. *Histopathology* 1991, **19**(5): 403-410.  
633
- 634 7. Esserman LJ, Moore DH, Tsing PJ, Chu PW, Yau C, Ozanne E, *et al.* Biologic  
635 markers determine both the risk and the timing of recurrence in breast cancer.  
636 *Breast Cancer Res Treat* 2011, **129**(2): 607-616.  
637
- 638 8. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human  
639 breast cancer: correlation of relapse and survival with amplification of the  
640 HER-2/neu oncogene. *Science* 1987, **235**(4785): 177-182.  
641
- 642 9. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ, *et al.*  
643 Meeting highlights: international expert consensus on the primary therapy of  
644 early breast cancer 2005. *Ann Oncol* 2005, **16**(10): 1569-1583.  
645
- 646 10. D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, *et al.*  
647 c-MYC induces mammary tumorigenesis by means of a preferred pathway  
648 involving spontaneous Kras2 mutations. *Nat Med* 2001, **7**(2): 235-239.  
649
- 650 11. Gunther EJ, Moody SE, Belka GK, Hahn KT, Innocent N, Dugan KD, *et al.*  
651 Impact of p53 loss on reversal and recurrence of conditional Wnt-induced  
652 tumorigenesis. *Genes Dev* 2003, **17**(4): 488-501.  
653
- 654 12. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, *et al.*  
655 Conditional activation of Neu in the mammary epithelium of transgenic mice  
656 results in reversible pulmonary metastasis. *Cancer Cell* 2002, **2**(6): 451-461.  
657
- 658 13. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, *et al.*  
659 The transcriptional repressor Snail promotes mammary tumor recurrence.  
660 *Cancer Cell* 2005, **8**(3): 197-209.  
661
- 662 14. Boxer RB, Jang JW, Sintasath L, Chodosh LA. Lack of sustained regression of  
663 c-MYC-induced mammary adenocarcinomas following brief or prolonged  
664 MYC inactivation. *Cancer Cell* 2004, **6**(6): 577-586.  
665

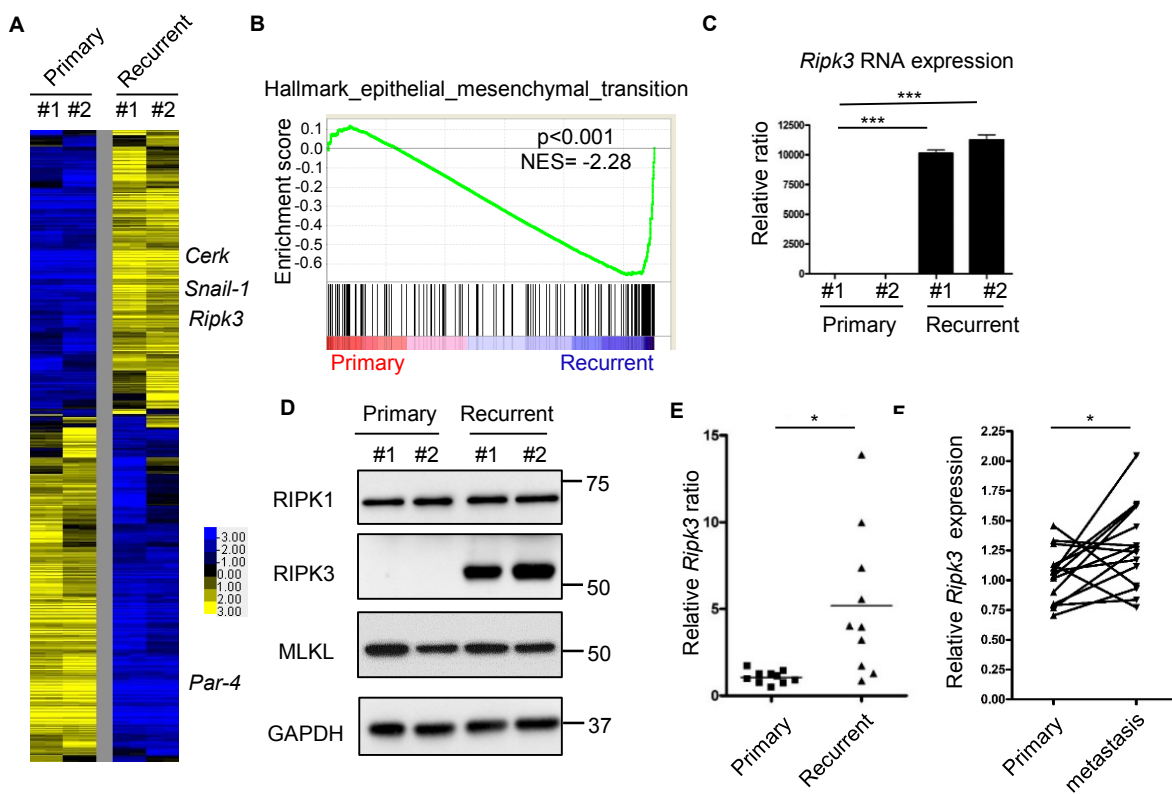
- 666 15. Wang Y, Zhou BP. Epithelial-mesenchymal Transition---A Hallmark of Breast  
667 Cancer Metastasis. *Cancer Hallm* 2013, **1**(1): 38-49.  
668
- 669 16. Tang X, Wu J, Ding CK, Lu M, Keenan MM, Lin CC, *et al.* Cystine Deprivation  
670 Triggers Programmed Necrosis in VHL-Deficient Renal Cell Carcinomas.  
671 *Cancer Res* 2016, **76**(7): 1892-1903.  
672
- 673 17. Tang X, Ding CK, Wu J, Sjol J, Wardell S, Spasojevic I, *et al.* Cystine  
674 addiction of triple-negative breast cancer associated with EMT augmented  
675 death signaling. *Oncogene* 2017, **36**(30): 4379.  
676
- 677 18. Lu SC. Regulation of glutathione synthesis. *Mol Aspects Med* 2009, **30**(1-2):  
678 42-59.  
679
- 680 19. Garcia-Ruiz C, Fernandez-Checa JC. Redox regulation of hepatocyte  
681 apoptosis. *J Gastroenterol Hepatol* 2007, **22 Suppl 1**: S38-42.  
682
- 683 20. Newton K. RIPK1 and RIPK3: critical regulators of inflammation and cell  
684 death. *Trends Cell Biol* 2015, **25**(6): 347-353.  
685
- 686 21. Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, *et al.* Mixed lineage kinase  
687 domain-like protein MLKL causes necrotic membrane disruption upon  
688 phosphorylation by RIP3. *Mol Cell* 2014, **54**(1): 133-146.  
689
- 690 22. Koo GB, Morgan MJ, Lee DG, Kim WJ, Yoon JH, Koo JS, *et al.* Methylation-  
691 dependent loss of RIP3 expression in cancer represses programmed necrosis  
692 in response to chemotherapeutics. *Cell Res* 2015, **25**(6): 707-725.  
693
- 694 23. Geserick P, Wang J, Schilling R, Horn S, Harris PA, Bertin J, *et al.* Absence of  
695 RIPK3 predicts necroptosis resistance in malignant melanoma. *Cell Death Dis*  
696 2015, **6**: e1884.  
697
- 698 24. Mabe NW, Fox DB, Lupo R, Decker AE, Phelps SN, Thompson JW, *et al.*  
699 Epigenetic silencing of tumor suppressor Par-4 promotes chemoresistance in  
700 recurrent breast cancer. *J Clin Invest* 2018, **128**(10): 4413-4428.  
701
- 702 25. Payne AW, Pant DK, Pan TC, Chodosh LA. Ceramide kinase promotes tumor  
703 cell survival and mammary tumor recurrence. *Cancer Res* 2014, **74**(21):  
704 6352-6363.  
705
- 706 26. Alvarez JV, Pan TC, Ruth J, Feng Y, Zhou A, Pant D, *et al.* Par-4  
707 downregulation promotes breast cancer recurrence by preventing  
708 multinucleation following targeted therapy. *Cancer Cell* 2013, **24**(1): 30-44.  
709
- 710 27. Karami-Tehrani F, Malek AR, Shahsavari Z, Atri M. Evaluation of RIP1K and  
711 RIP3K expressions in the malignant and benign breast tumors. *Tumour Biol*  
712 2016, **37**(7): 8849-8856.  
713

- 714 28. Vecchi M, Confalonieri S, Nuciforo P, Vigano MA, Capra M, Bianchi M, *et al.*  
715 Breast cancer metastases are molecularly distinct from their primary tumors.  
716 *Oncogene* 2008, **27**(15): 2148-2158.  
717
- 718 29. Mathe A, Wong-Brown M, Morten B, Forbes JF, Braye SG, Avery-Kiejda KA,  
719 *et al.* Novel genes associated with lymph node metastasis in triple negative  
720 breast cancer. *Sci Rep* 2015, **5**: 15832.  
721
- 722 30. Sun L, Wang H, Wang Z, He S, Chen S, Liao D, *et al.* Mixed lineage kinase  
723 domain-like protein mediates necrosis signaling downstream of RIP3 kinase.  
724 *Cell* 2012, **148**(1-2): 213-227.  
725
- 726 31. Normand G, King RW. Understanding cytokinesis failure. *Adv Exp Med Biol*  
727 2010, **676**: 27-55.  
728
- 729 32. Ganem NJ, Cornils H, Chiu SY, O'Rourke KP, Arnaud J, Yimlamai D, *et al.*  
730 Cytokinesis failure triggers hippo tumor suppressor pathway activation. *Cell*  
731 2014, **158**(4): 833-848.  
732
- 733 33. Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation  
734 of p53 alleviates inhibition by MDM2. *Cell* 1997, **91**(3): 325-334.  
735
- 736 34. Giam M, Rancati G. Aneuploidy and chromosomal instability in cancer: a  
737 jackpot to chaos. *Cell Div* 2015, **10**: 3.  
738
- 739 35. Taylor AM, Shih J, Ha G, Gao GF, Zhang X, Berger AC, *et al.* Genomic and  
740 Functional Approaches to Understanding Cancer Aneuploidy. *Cancer Cell*  
741 2018, **33**(4): 676-689 e673.  
742
- 743 36. Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer.  
744 *Cancer Cell* 2016, **29**(6): 783-803.  
745
- 746 37. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, *et al.* Inactivation of YAP  
747 oncoprotein by the Hippo pathway is involved in cell contact inhibition and  
748 tissue growth control. *Genes Dev* 2007, **21**(21): 2747-2761.  
749
- 750 38. Yang Z, Nakagawa K, Sarkar A, Maruyama J, Iwasa H, Bao Y, *et al.*  
751 Screening with a novel cell-based assay for TAZ activators identifies a  
752 compound that enhances myogenesis in C2C12 cells and facilitates muscle  
753 repair in a muscle injury model. *Mol Cell Biol* 2014, **34**(9): 1607-1621.  
754
- 755 39. Poursaitidis I, Wang X, Crighton T, Labuschagne C, Mason D, Cramer SL, *et al.*  
756 Oncogene-Selective Sensitivity to Synchronous Cell Death following  
757 Modulation of the Amino Acid Nutrient Cystine. *Cell Reports* 2017, **18**(11):  
758 2547-2556.  
759
- 760 40. Lewerenz J, Klein M, Methner A. Cooperative action of glutamate transporters  
761 and cystine/glutamate antiporter system Xc<sup>-</sup> protects from oxidative glutamate  
762 toxicity. *J Neurochem* 2006, **98**(3): 916-925.  
763

- 764 41. Dolma S, Lessnick SL, Hahn WC, Stockwell BR. Identification of genotype-  
765 selective antitumor agents using synthetic lethal chemical screening in  
766 engineered human tumor cells. *Cancer Cell* 2003, **3**(3): 285-296.  
767
- 768 42. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE,  
769 *et al.* Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*  
770 2012, **149**(5): 1060-1072.  
771
- 772 43. Gout PW, Buckley AR, Simms CR, Bruchovsky N. Sulfasalazine, a potent  
773 suppressor of lymphoma growth by inhibition of the x(c)- cystine transporter: a  
774 new action for an old drug. *Leukemia* 2001, **15**(10): 1633-1640.  
775
- 776 44. Skouta R, Dixon SJ, Wang J, Dunn DE, Orman M, Shimada K, *et al.*  
777 Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease  
778 models. *J Am Chem Soc* 2014, **136**(12): 4551-4556.  
779
- 780 45. Wang K, Li J, Degterev A, Hsu E, Yuan J, Yuan C. Structure-activity  
781 relationship analysis of a novel necroptosis inhibitor, Necrostatin-5. *Bioorg*  
782 *Med Chem Lett* 2007, **17**(5): 1455-1465.  
783
- 784 46. Liu X, Zhou M, Mei L, Ruan J, Hu Q, Peng J, *et al.* Key roles of necroptotic  
785 factors in promoting tumor growth. *Oncotarget* 2016, **7**(16): 22219-22233.  
786
- 787 47. Seifert L, Werba G, Tiwari S, Giao Ly NN, Alothman S, Alqunaibit D, *et al.* The  
788 necrosome promotes pancreatic oncogenesis via CXCL1 and Mincle-induced  
789 immune suppression. *Nature* 2016, **532**(7598): 245-249.  
790
- 791 48. Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R,  
792 Viswanathan VS, *et al.* Regulation of ferroptotic cancer cell death by GPX4.  
793 *Cell* 2014, **156**(1-2): 317-331.  
794
- 795 49. Chen MS, Wang SF, Hsu CY, Yin PH, Yeh TS, Lee HC, *et al.* CHAC1  
796 degradation of glutathione enhances cystine-starvation-induced necroptosis  
797 and ferroptosis in human triple negative breast cancer cells via the GCN2-  
798 eIF2alpha-ATF4 pathway. *Oncotarget* 2017, **8**(70): 114588-114602.  
799
- 800 50. Cramer SL, Saha A, Liu J, Tadi S, Tiziani S, Yan W, *et al.* Systemic depletion  
801 of L-cyst(e)ine with cyst(e)inase increases reactive oxygen species and  
802 suppresses tumor growth. *Nat Med* 2017, **23**(1): 120-127.  
803
- 804 51. Yoshikawa M, Tsuchihashi K, Ishimoto T, Yae T, Motohara T, Sugihara E, *et*  
805 *al.* xCT inhibition depletes CD44v-expressing tumor cells that are resistant to  
806 EGFR-targeted therapy in head and neck squamous cell carcinoma. *Cancer*  
807 *Res* 2013, **73**(6): 1855-1866.  
808
- 809 52. Lin CC, Kitagawa M, Tang X, Hou MH, Wu J, Qu DC, *et al.* CoA synthase  
810 regulates mitotic fidelity via CBP-mediated acetylation. *Nat Commun* 2018,  
811 **9**(1): 1039.  
812

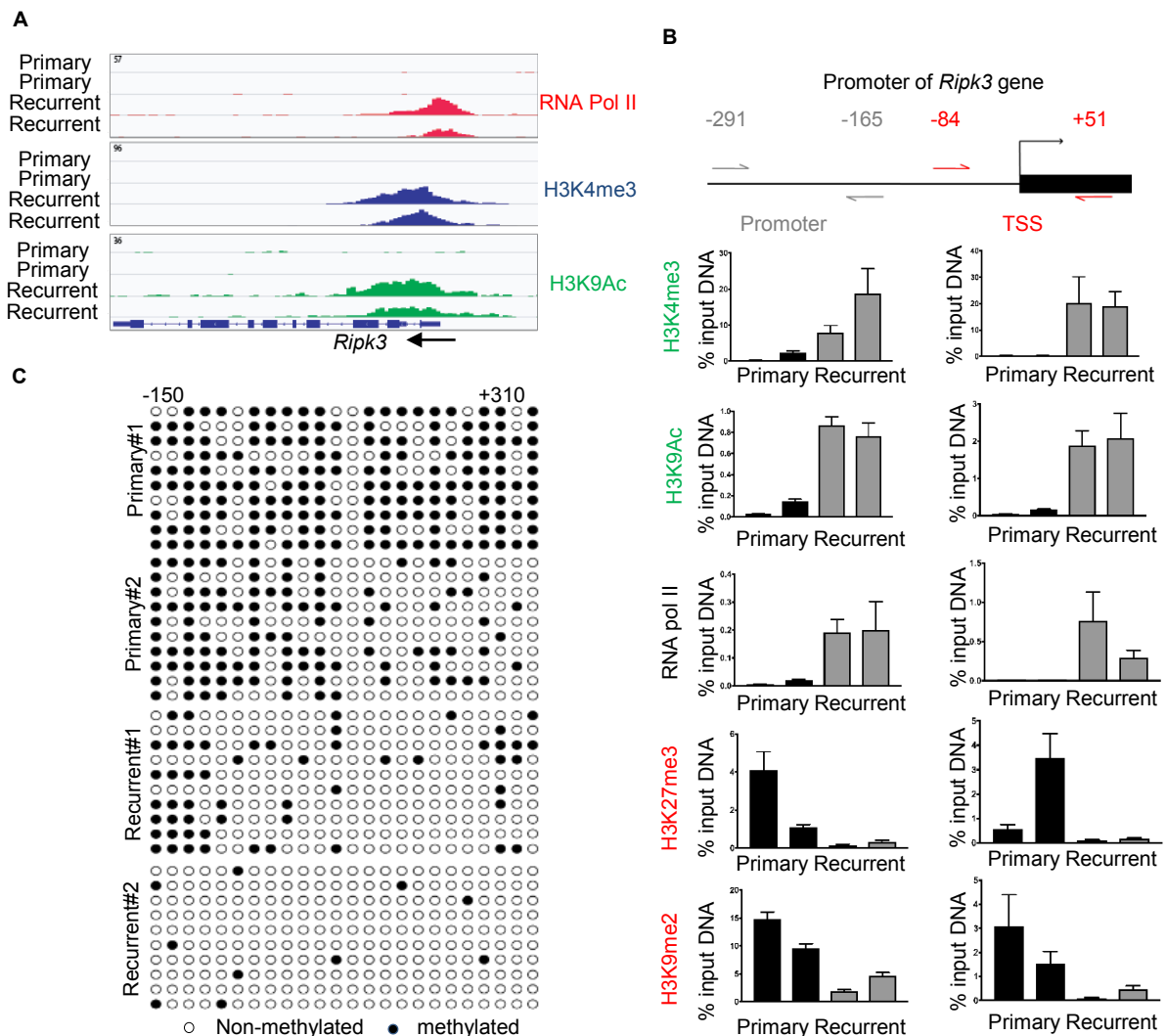
- 813 53. Yang Z, Jiang B, Wang Y, Ni H, Zhang J, Xia J, *et al.* 2-HG Inhibits  
814 Necroptosis by Stimulating DNMT1-Dependent Hypermethylation of the RIP3  
815 Promoter. *Cell Rep* 2017, **19**(9): 1846-1857.  
816  
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**Figure 1**



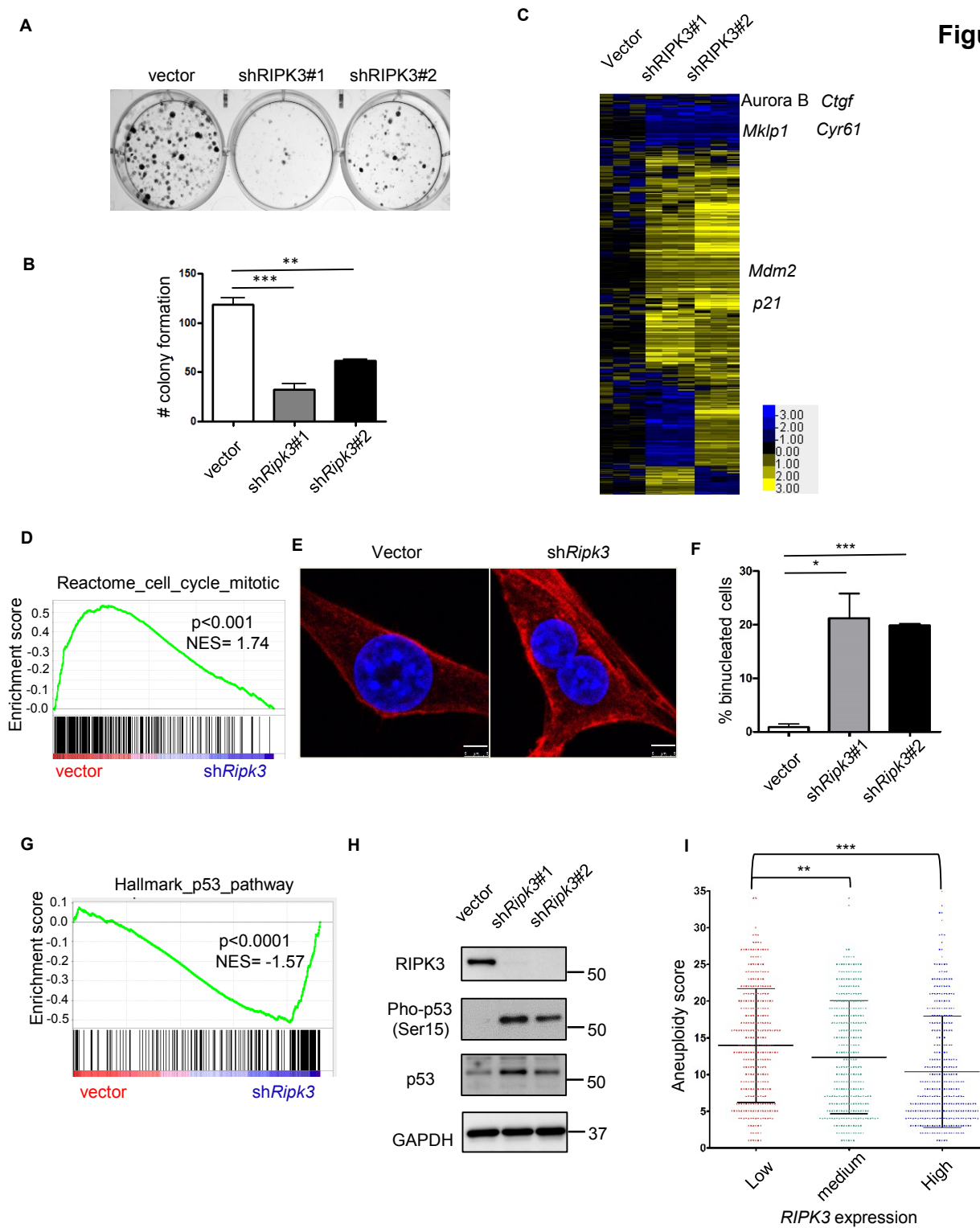


**Figure 2**

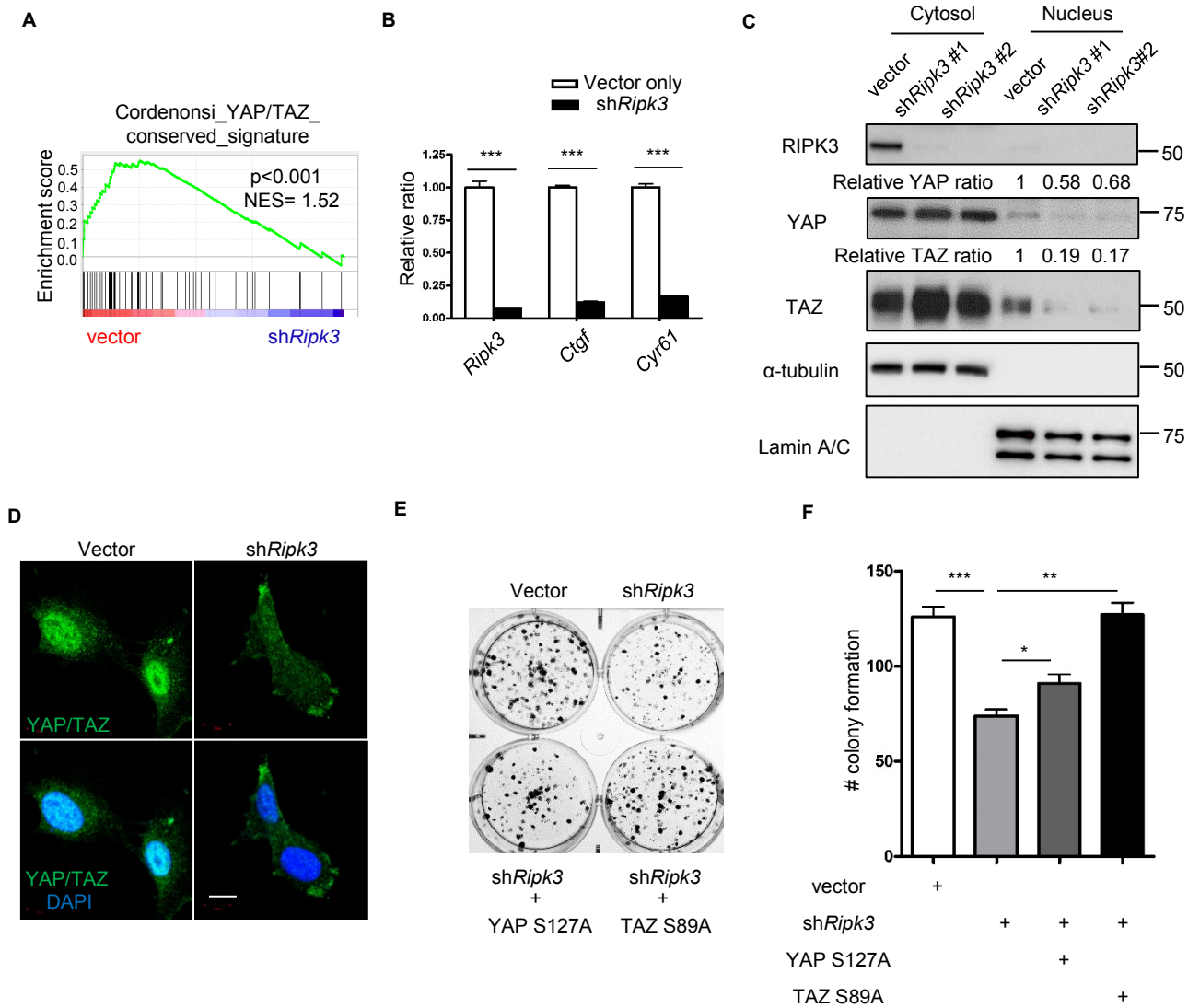




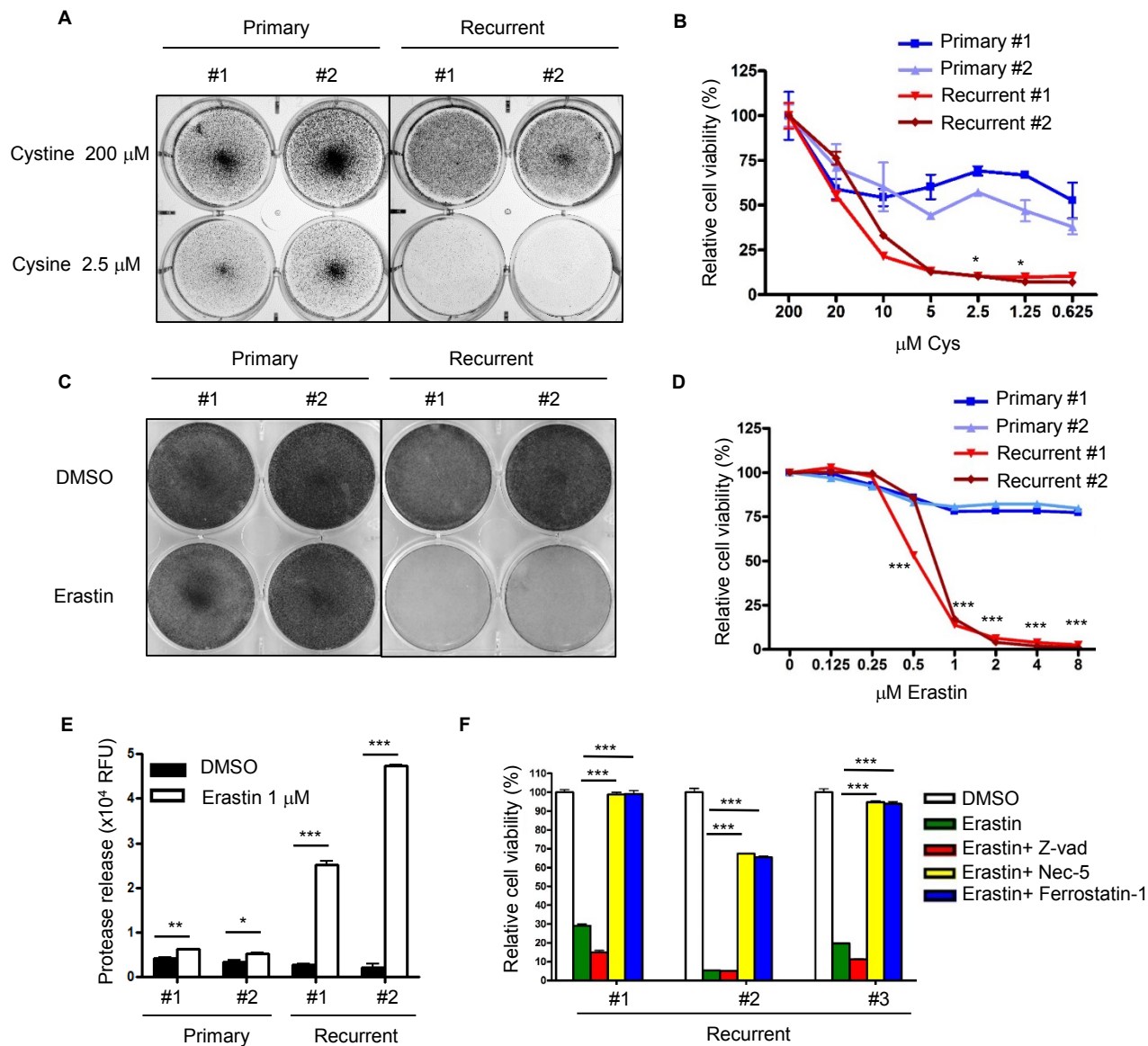
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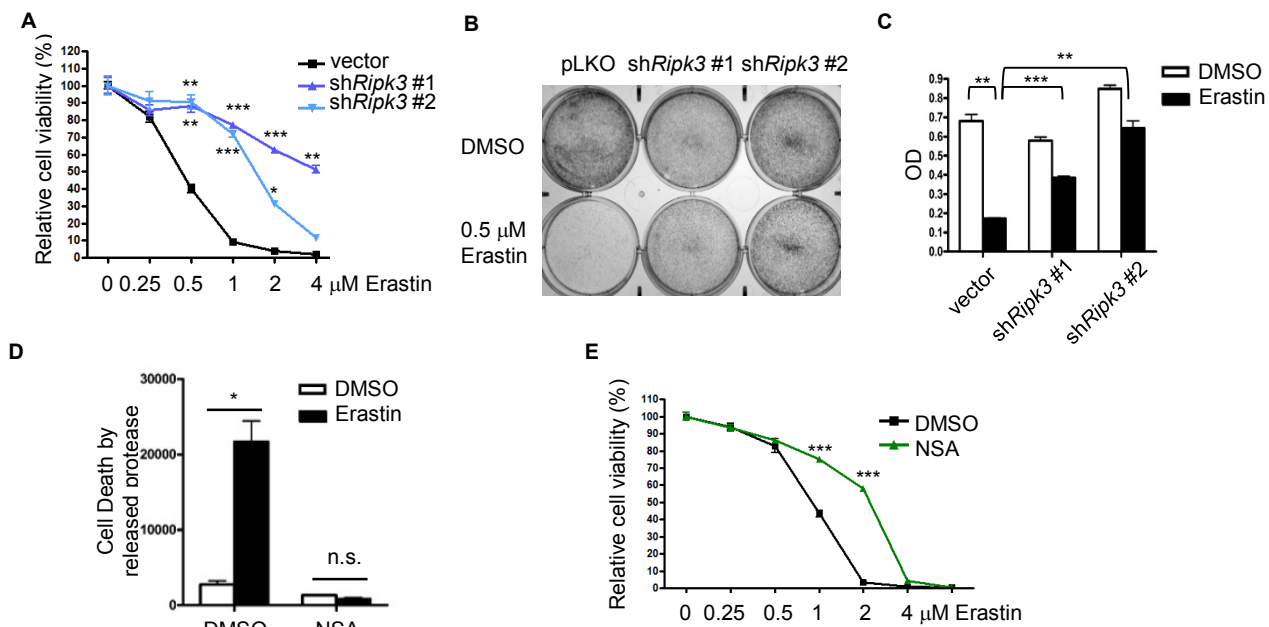
**Figure 4**



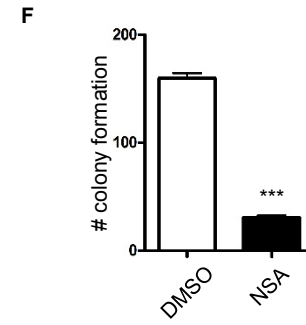
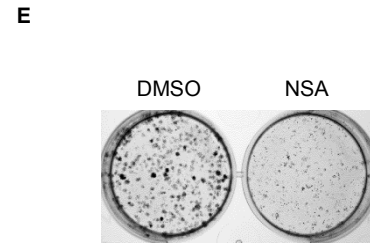
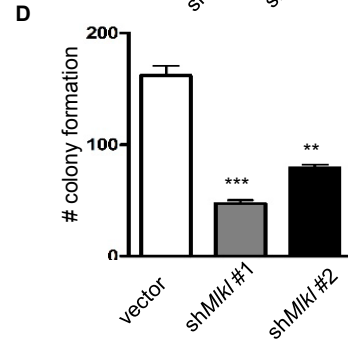
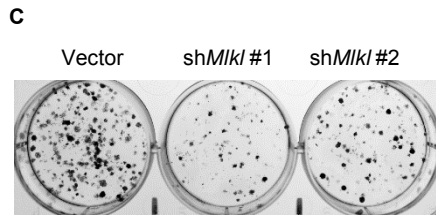
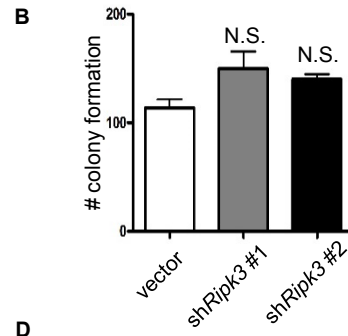
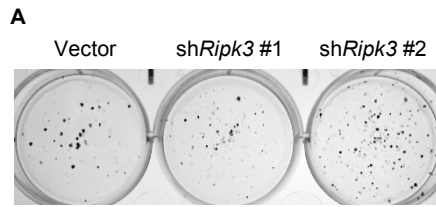
**Figure 5**



**Figure 6**

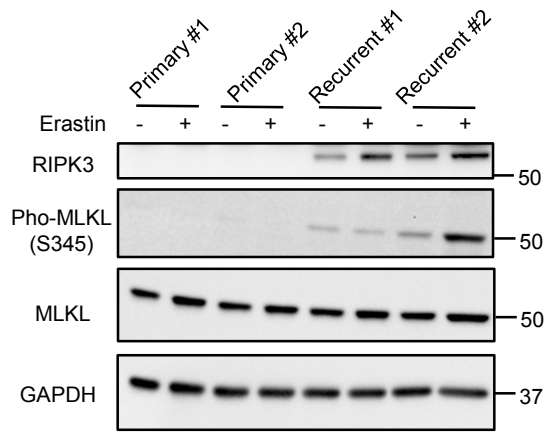


# Supplemental Figure 1

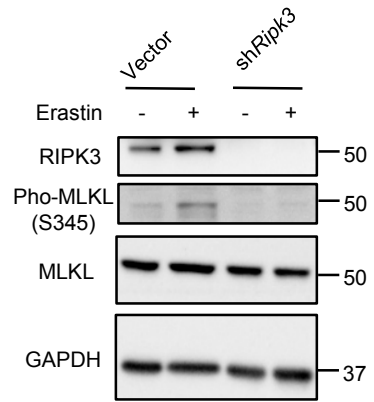


## Supplemental Figure 2

**A**



**B**



## Supplemental Table 1

Gene name	Probe Set	Genbank	fold change in metastases vs tumors	Lymph node Metastasis	Primary breast Tumor	Anova P-value
RIPK3	228139_at	NM_006871	2.08	1.33	0.64	1.53E-02