

Polyploid cancer cells reveal signatures of chemotherapy resistance

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45 **Abstract**

46 Therapeutic resistance in cancer significantly contributes to
47 mortality, with many patients eventually experiencing recurrence
48 after initial treatment responses. Recent studies have identified
49 therapy-resistant large polyploid cancer cells in patient tissues,
50 particularly in late-stage prostate cancer, linking them to advanced
51 disease and relapse. Here, we analyzed bone marrow aspirates
52 from 44 advanced prostate cancer patients and found the
53 presence of circulating tumor cells with increased genomic content
54 (CTC-IGC) was significantly associated with poorer progression-
55 free survival. Single cell copy number profiling of CTC-IGC
56 displayed clonal origins with typical CTCs, suggesting complete
57 polyploidization. Induced polyploid cancer cells from PC3 and
58 MDA-MB-231 cell lines treated with docetaxel or cisplatin were
59 examined through single cell DNA sequencing, RNA sequencing,
60 and protein immunofluorescence. Novel RNA and protein
61 markers, including HOMER1, TNFRSF9, and LRP1, were
62 identified as linked to chemotherapy resistance. These markers
63 were also present in a subset of patient CTCs and associated with
64 recurrence in public gene expression data. This study highlights
65 the prognostic significance of large polyploid tumor cells, their role
66 in chemotherapy resistance, and their expression of markers tied
67 to cancer relapse, offering new potential avenues for therapeutic
68 development.

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70

71 **Keywords**

72 polyploid giant cancer cell; circulating tumor cell; progression-free
73 survival; liquid biopsy; polyan euploid cancer cell state;
74 chemotherapy resistance; single cell

75

76 **1. Introduction**

77 While initial treatment efficacy is observed in most patients with
78 prostate or breast cancer, prostate cancers recur in 24-48% of
79 cases [1], and breast cancers relapse in about 30% of patients [2-
80 3]. In general, late-stage metastatic cancers are more difficult to
81 control, and patients are typically treated with chemotherapy;
82 unfortunately, complete response rates from chemotherapy
83 treatments in patients with late stage disease are low [4-5].
84 Despite defining numerous detailed intrinsic and extrinsic
85 mechanisms that enable cancer cell survival under therapy,
86 therapy resistance remains responsible for over 90% of cancer
87 related deaths [6-8].

88 Large polyploid tumor cells are correlated with late disease
89 stages, poor prognosis, and therapy resistance across virtually
90 every tumor type [9-13]. Large polyploid tumor cells are induced
91 through various stressors, including common chemotherapies
92 such as docetaxel and cisplatin [14-17]. Evidence has shown that
93 whole genome doubling (WGD) events and altered ploidy levels
94 are poor prognostic indicators across cancer types and are

95 ultimately thought to provide cancer cells the ability to evolve and
96 survive therapy [18-21].

97 Recent studies have shown that large polyploid tumor cells
98 can give rise to viable progeny that display more malignant and
99 stem cell characteristics than the parental population they
100 descended from [22]. Importantly, targeting identified pathways,
101 including AP-1, HIF2a, cholesterol-related, and embryogenic-
102 related pathways, reduced the number of surviving large polyploid
103 cancer cells, as well as surviving progeny cells following therapy
104 [22-26]. While significant, these studies lack single-cell molecular
105 resolution and note that not all cells are eliminated. What
106 ultimately matters is that some cancer cells are still capable of
107 survival and result in disease progression. Identification of novel
108 biomarkers that can predict patients' recurrence and resistance to
109 therapy may lead to better treatment outcomes.

110 We find that the presence of circulating tumor cells (CTCs)
111 with increased genomic content in the bone marrow aspirate of
112 late-stage prostate cancer patients is significantly associated with
113 worse progression free survival. We comprehensively evaluated
114 large polyploid tumor cells (prostate cancer PC3 and breast
115 cancer MDA-MB-231) that survive following treatment with two
116 chemotherapy classes (cisplatin and docetaxel), and functionally
117 characterize the surviving cells through a multi-omic approach,
118 including morphometric, genomic, and transcriptomic profiling at
119 the single cell level. We find that progeny cells differed
120 substantially from the original parental population and most

121 closely resembled the transcriptome of the large polyploid tumor
 122 cells from which they were derived. We also find novel markers
 123 associated with chemotherapy survival are upregulated in cells
 124 that survive treatment, are retained in the progeny from surviving
 125 cells, and are significantly associated with recurrence in prostate
 126 and breast cancer at the RNA level. These novel survival
 127 biomarkers are expressed at the protein level in the CTCs of
 128 patients who also have recurrent disease. Taken together, our
 129 results highlight novel biomarkers of survival and shed light on the
 130 functionality of large polyploid tumor cells and their role in disease
 131 recurrence.

132

133 **2. Methods**

134 **Patient sample collection and processing:** Liquid biopsy
 135 samples were collected from clinical sites and processed at the
 136 University of Southern California as previously described [27-28].
 137 Briefly, peripheral blood (PB) and bone marrow aspirate (BM)
 138 samples were collected from patients immediately starting
 139 treatment on trial NCT01505868 that evaluated cabazitaxel with or
 140 without carboplatin in patients with metastatic castration-resistant
 141 prostate cancer. Samples were collected at MD Anderson Cancer
 142 Center prior to therapy.

143 Patients 1 and 3 did not participate in NCT01505868.

144 Patient 1, a previous case study, was acquired from the Greater
 145 Los Angeles Veterans' Affairs Healthcare System [29]. The bone

146 marrow sample was collected at the time of diagnostic biopsy,
147 prior to treatment. Patient 3, another previous case study [30],
148 was acquired from MD Anderson. All patients gave written
149 informed consent in accordance with approved institutional review
150 board and research development (VA) protocols.

151 Following isotonic erythrocyte lysis, the entire nucleated
152 fraction was plated onto custom cell adhesion glass slides
153 (Marienfield, Lauda, Germany) and stored at -80°C until use [28].

154 **Cell culture and drug treatment:** PC3 and MDA-MB-231 cell
155 lines were purchased from ATCC and grown in RPMI and DMEM,
156 respectively, with 10% FBS and 0.5% penicillin / streptavidin. Cells
157 were plated at a density of 625,000 cells per T-75 flask. PC3 cells
158 were treated with 5 nM docetaxel (PC3: 5nM, MDA-MB-221:
159 10nM) or cisplatin (10 µM) for 72 hours. Cells were then allowed
160 to recover in normal medium for 1 or 10 days. When indicated,
161 PC3 cells were re-treated at day 10 post treatment removal. Cells
162 were lifted from culture and plated on Marienfield glass slides for
163 imaging or single cell isolation. All cell line experiments were
164 conducted in triplicate.

165 To isolate progeny cells, PC3 cells 10 days post treatment
166 were lifted with 1x versene. Biosorter (UnionBio, Holliston, MA)
167 was used to sort single cells based on size and the largest 15% of
168 cells were sorted into ten 96-well plates (n=960 individual wells)
169 containing RPMI medium and then placed in a 37°C incubator.
170 Media was changed every 2-3 days.

171 **Immunofluorescent staining:** Patient slides in Figure 1 were
172 fixed with paraformaldehyde and stained with a pan-cytokeratin
173 cocktail mixture (see supplementary methods), conjugated mouse
174 anti-human CD45 Alexa Fluor 647 (clone: F10-89-4, MCA87A647,
175 AbD Serotec, Raleigh, NC, USA), Vimentin (Alexa Fluor 488 rabbit
176 IgG monoclonal antibody (Cell Signaling Technology; Cat#
177 9854BC; Clone: D21H), and 4',6-diamidino-2-phenylindole (DAPI;
178 D1306, Thermo, Waltham, MA, USA) as previously described [28].
179 EpCAM (Thermo, 14-9326-82) was included in the pan-cytokeratin
180 cocktail mixture to make an “EPI-cocktail”.
181 TNFRSF9 (Thermo, PA5-98296) and HOMER1 (Thermo,
182 PA5-21487) primary antibodies were incubated on slides overnight
183 at 4°C with the EPI-cocktail of antibodies. Slides were then
184 washed and incubated at room temperature for two hours with
185 Alexa Fluor 555 goat anti-mouse IgG1 antibody (Thermo,
186 A21127), Alexa Fluor 488 goat anti-rabbit (Thermo, A11034),
187 CD45, and DAPI.
188 LRP1 (Thermo, 377600) was generated in mice and was
189 therefore not compatible with the EPI-cocktail. Instead, LRP1 was
190 incubated overnight at 4°C. Slides were then washed and
191 incubated at room temperature for two hours with Alexa Fluor 555
192 goat anti-mouse IgG1 antibody. Next, pre-conjugated Alexa Fluor
193 488 pan-cytokeratin (53-9003-82, Thermo) recognizing CK 10, 14,
194 15, 16, and 19 was incubated with conjugated mouse anti-human
195 CD45, and DAPI.

196 **Slide imaging and analysis:** Slides were imaged with an
 197 automated high throughput microscope equipped with a 10x
 198 optical lens, as previously described [27]. Immunofluorescent and
 199 bright field images were collected. Image analysis tool, available
 200 at <https://github.com/aminnaghdloo/slide-image-utils>, was
 201 developed in python using the OpenCV and scikit-image
 202 packages [31-32]. Briefly, each fluorescent channel was
 203 segmented individually using adaptive thresholding and merged
 204 into one cell mask. Cell mask and DAPI mask were used to
 205 extract features and fluorescent intensity statistics of single cells
 206 and their nuclei, respectively. For nucleus size analysis, equivalent
 207 diameter was calculated from nucleus area, assuming a circular
 208 shape.

209 **Fluorescence *in situ* Hybridization:** Probes for centromeres of
 210 chromosomes 1 (CHR01-10-GR) and 10 (CHR10-10-GR) were
 211 purchased from Empire Genomics (Depew, New York) and the
 212 hybridization was carried out on Marienfeld glass slides per the
 213 manufacturer's instructions. Slides were then stained with DAPI
 214 and then imaged.

215 **Single cell copy number profiling:** Single cells were isolated as
 216 previously described [28]. Copy number profiling from low pass
 217 whole genome sequencing samples was conducted as previously
 218 described (see supplementary methods) [33-34].

219 **Single cell RNA sequencing:** Single cells were isolated and
 220 picked via micro-manipulation as previously described. RNA was
 221 extracted via a modified Smart-Seq2 approach and library

222 prepped with Nextera XT (Illumina, San Diego, CA). Cells were
223 sequenced paired end by 150 base-pairs on an Illumina HiSeq
224 4000 (Fulgent). Read adapters were trimmed with TrimGalore
225 (version 0.6.7) and aligned with the HiSat2 (v2.2.1). Picard
226 (v3.0.0) was used to visualize RNA mapping quality control [35-
227 37]. HTSeq (v2.0.2) was used to generate a gene count matrix
228 [38].

229 The SingleCellExperiment package (v4.2.2) was utilized for
230 inputting count data into downstream analyses, such as
231 converting to Seurat (v4.3.0) and edgeR (v3.36.0) count matrices
232 [39]. Downstream analysis was performed with R (v4.1.2). Data
233 visualization was performed with Seurat and ggplot2 (v3.4.4), and
234 Pheatmap (v1.0.12) packages.

235 The edgeRQLFDetRate differential expression pipeline
236 was used to find common upregulated genes in polyploid cancer
237 cells [40]. Sequencing batches were controlled for. Shared genes
238 expressed in surviving large cells were intersected through R.

239 Gene datasets were downloaded directly from CHEA3 [41]
240 and MSigDB [42] for transcription factor and hallmark pathway
241 enrichment, respectively. Single cell enrichment was conducted
242 through JASMINE [43].

243

244 **Survival analysis:** Survival analysis from patient bone marrow
245 and peripheral blood samples was performed with the Survival R
246 package (v3.5.5) and plotted with ggplot2 (v3.4.4). Public gene
247 expression survival analysis was analyzed via PanCancSurvPlot

248 [44] for prostate cancer (GSE116918) and breast cancer
249 (GSE10893) [45-46].

250

251 **3. Results**

252 **CTCs with increased genomic content (CTC-IGC) are found in**
253 **the bone marrow of late-stage prostate cancer patients and**
254 **are correlated with worse progression free survival**

255 Liquid biopsies from peripheral blood and bone marrow
256 aspirate were acquired from a late-stage prostate cancer cohort
257 (NCT01505868). Matched bone marrow and peripheral blood
258 samples from 31 patients were analyzed for CTCs. CTCs with
259 increased genomic content (CTC-IGC), identified as having a
260 nuclear diameter at least double the average of the CTC cell
261 population, were found in 9.7% of peripheral blood samples. CTC-
262 IGC were present in 80.6% of bone marrow samples from the
263 same patients (Fig. 1A-B, S1). Survival analysis with 44 bone
264 marrow samples (from the 31 patients with matched blood
265 samples and 13 patients without matched blood) showed that
266 presence of at least one CTC-IGC detected in the bone marrow
267 was associated with decreased progression-free survival (Fig. 1C,
268 Table S1). Previous treatment history was available for 33 of the
269 44 patients and primarily included anti-androgens and other
270 hormonal treatments (i.e., bicalutamide, nilutamide,
271 enzalutamide). The six patients who were previously treated with
272 docetaxel were all positive for CTCs-IGC in the bone marrow.

273 Clonal tumor lineage measured via copy number ratio
274 analysis was confirmed in both typical CTCs and CTCs-IGC. No
275 apparent differences in copy number ratios were identified
276 between the two CTC groups (Fig. 1D-F, S2, S3). These
277 observations show that CTC-IGC can be found in blood and bone
278 marrow aspirate, are tumor derived, and thus may contribute
279 towards relapse in late-stage prostate cancer. Despite the
280 apparent WGD of CTC-IGC, these cells retain the original tumor
281 copy number profile. To understand the importance and behavior
282 of this phenotype, we used an *in vitro* model of polyploid tumor
283 cells to investigate their relationship with therapeutic resistance.

284

285 **Large polyploid cancer cells form as a response to** 286 **chemotherapy in prostate and breast cancer models**

287 PC3 and MDA-MB-231 cells were treated with sublethal
288 doses of docetaxel or cisplatin for 72 hours. Following
289 chemotherapy, cells were allowed to recover for 1 or 10 days in
290 their regular growth medium, lifted from culture, plated on
291 specialized glass slides, stained with cell and nuclear markers,
292 then imaged through high content scanning and evaluated for
293 nuclear size and other morphometric comparisons (Fig. 2A). While
294 there was significant cell death as expected (Fig. S4A), surviving
295 cells increased in both nuclear diameter and cell size as a function
296 of time (Fig. 2B-E; Fig. S4B).

297 To evaluate resistance, we treated cells that survived
298 cisplatin treatment (10 days post treatment; 10 DPT) with cisplatin

299 or docetaxel. Compared to the control condition (initially cisplatin
300 treated and then re-treated with DMSO) cell counts and cell
301 viability were not significantly impacted, suggesting that these
302 cells are not sensitive to additional rounds of chemotherapy (Fig.
303 S4A, S4D, 2E).

304 To obtain progeny cells from a single chemotherapy-
305 induced surviving polyploid cell, we isolated and single-cell
306 seeded PC3 cells 10 days post-cisplatin release (n=480) and 10
307 days post-docetaxel release (n=960) and monitored for colony
308 formation. From these, only 2 polyploid docetaxel-treated PC3
309 cells gave rise to progeny after 2 months (progeny-1) and 2.5
310 months (progeny-2). Progeny-2 failed to proliferate following the
311 first passage. Over the course of the three-month experiment,
312 approximately 50% of the polyploid cells treated with either
313 cisplatin or docetaxel remained viable and adherent. The dividing
314 progeny-1 cells displayed a larger nuclear and cellular diameter
315 than the parental PC3 population from which it originated (Fig. 2F,
316 S4). We treated progeny-1 with docetaxel or cisplatin and found
317 that the population was sensitive to both chemotherapies. Further,
318 following 10 days of recovery, surviving progeny-1 cells had
319 increased nuclear and cell diameter, similar to what was observed
320 from the original parent population (Fig. 2F, S4B).

321 **Surviving PC3 polyploid cancer cells show no additional copy** 322 **number ratio alterations compared to parental controls**

323 To evaluate the presence of genomic alterations in the
324 surviving polyploid cells and their progeny, we assayed copy

325 number status and cell ploidy. Strikingly, surviving large polyploid
 326 PC3 and MDA-MB-231 cells from both docetaxel and cisplatin
 327 treatments showed no apparent copy number ratio differences
 328 compared to control cells (Fig. 3A-C, S5). This result confirms
 329 patient data in that copy number ratio status does not differ
 330 between CTCs with normal nuclei and CTCs with larger nuclei
 331 (Fig. 1D-F) and suggests that cells are undergoing complete WGD
 332 rather than displaying specific copy number breakpoints. While
 333 copy number status did display minor differences in the progeny-1
 334 compared to parental control (e.g., an increased 3p gain) (Fig. 3A-
 335 B), no substantial alterations were observed. Conversely, progeny-
 336 2, the clone that did not survive the first passage, displayed the
 337 most aberrant copy number profile compared to the other
 338 conditions (i.e., 6 gain and 4p gain) and clustered separately from
 339 the other PC3 cell conditions (Fig. 3A).

340 FISH probes for the centromeres of PC3 chromosome 1
 341 (ploidy = 3) and chromosome 10 (ploidy = 1) showed no
 342 statistically significant differences when comparing DMSO
 343 parental control cells to progeny-1 cells (Fig. 3D), suggesting that
 344 any apparent scars of ploidy reduction were not present. These
 345 results prompted investigation into the phenotype of these
 346 surviving cells.

347 **Single cell transcriptomic profiling reveals common genes**
 348 **and pathways upregulated in PC3 and MDA-MB-231 polyploid**
 349 **cells**

350 497 PC3 cells were isolated and sequenced in 5 separate
351 batches (Fig. S6) and included: DMSO control (n=129), 1-day
352 post-cisplatin release (n=78), 10 days post-cisplatin release
353 (n=68), 1-day post-docetaxel release (n=45), 10 days post-
354 docetaxel release (n=118), docetaxel progeny-1 (n=12), docetaxel
355 progeny-2 (n=13). Two batches of 203 MDA-MB-231 cells
356 included: DMSO control (n=43), 1-day post-cisplatin release
357 (n=22), 10 days post-cisplatin release (n=62), 1-day post-
358 docetaxel release (n=24), 10 days post-docetaxel release (n=62)
359 (Fig. S7).

360 Regardless of treatment, a general spatial separation that
361 was dependent on recovery duration was observed in PC3 and
362 MDA-MB-231 cells (Fig. 4A, S8). To identify convergent
363 phenotypes regardless of tumor type or therapy, we evaluated
364 genes that were upregulated in both PC3 and MDA-MB-231
365 following either cisplatin or docetaxel treatment. MDA-MB-231
366 cells 10 days post cisplatin or docetaxel release upregulated 1591
367 shared genes compared to DMSO control; PC3 cells 10 days post
368 cisplatin or docetaxel treatment upregulated 1178 shared genes
369 compared to DMSO control (LFC > 1.5, FDR < 0.01; Fig. 4B).
370 Intersection of the shared gene sets showed MDA-MB-231 and
371 PC3 cells that survive either cisplatin or docetaxel exposure
372 shared 309 upregulated genes (Fig. 4B; Table S2). The 309
373 shared genes were considered a survivor cell enrichment data set,
374 which was further evaluated.

375 Of the 309 shared genes, 77% were protein coding and
376 17% were lncRNAs, while the remaining ~6% were pseudogenes
377 or yet to be experimentally confirmed (TEC, not yet tested; Fig.
378 4C). Log-fold change values were plotted for PC3-Doc-DPT10 vs
379 MDA-Doc-DPT10 (Fig. 4D) and PC3-Cis-DPT10 vs MDA-Cis-
380 DPT10 (Fig. 4E). Within each treatment class, shared differentially
381 expressed genes (DEGs) were positively correlated between
382 MDA-MB-231 and PC3 cells, indicating the DEGs are upregulated
383 to a similar magnitude.

384 Common transcription factors (TFs) and hallmark
385 pathways upregulated in the survivors were delineated (Fig. 4F-
386 G). Two significantly enriched TFs, ZNF697 and NPAS2, were
387 previously reported in cells that transition out of senescence and
388 into a proliferative state [47]. Top enriched hallmark pathways in
389 the 309 gene survivor data set were: epithelial-to-mesenchymal
390 transition (EMT), upregulation of KRAS signaling, coagulation,
391 TNF α signaling via NF κ B, and hypoxia (Fig. 4F). Single cell gene
392 enrichment confirmed the top upregulated hallmark pathways in
393 the shared survivor data set (Fig. 4H, S8-9). Additional pathways
394 identified to be significantly upregulated in the surviving cells
395 were: PI3K-AKT-mTOR Signaling, Inflammatory Response, and
396 Cholesterol Homeostasis (Fig. 4H, S8-9).

397

398 **Identification of HOMER1, TNFRSF9, and LRP1 as putative**
399 **chemotherapy RNA survival markers**

400 Utilizing the shared cell survivor gene set data, markers
401 were independently evaluated to understand their putative role in
402 chemotherapy survival and polyploid state. All 309 genes were
403 investigated via literature review and queried for terms in
404 September 2023, including: large tumor cell, polyploid giant
405 cancer cell, poly-aneuploid cancer cell, survival pathways, drug
406 resistance, chemotherapy, and apoptosis. With prior knowledge
407 that top upregulated genes (MMP-3, SAA1, and C3) functioned in
408 the execution of apoptosis and clearance of apoptotic bodies (Fig.
409 4D-E), and that SAA1 and C3 were correlated with better PFS
410 (Fig. S10), they were not considered novel survival markers. The
411 309 gene survivor cell enrichment data set was also intersected
412 with genes in the top enriched pathways that modulate survival:
413 TNFa via NFkB, PI3K-AKT, and mTOR signaling (Fig. 4G-H). We
414 identified TNFRSF9 and LRP1 as survival biomarkers; these are
415 known to function as cell surface receptors that enhance PI3K
416 activity. This activity, in turn, stimulates AKT, thereby promoting
417 cell survival (Fig. 4D-E, Fig. 5, S11A) [48-50]. Further, we
418 identified HOMER1 as a PC3-specific survival marker (Fig. 5);
419 HOMER1 plays a role in mTOR signaling and protection against
420 apoptosis [51-54].
421
422 **HOMER1, TNFRSF9, and LRP1 are protein markers of**
423 **chemotherapy survival and are retained in docetaxel treated**
424 **PC3 progeny**

425 At the protein level, we found surviving PC3 and MDA-MB-
426 231 cells post-chemotherapy treatment stained positive for
427 HOMER1, TNFRSF9, and LRP1 (Fig. 5A, 5D). Image
428 quantification revealed all PC3 conditions (except for progeny-1
429 cisplatin day 10 post-treatment release) were significantly
430 upregulated compared to controls (Fig 5A, 5C). Day 10 survivors
431 showed the highest protein expression levels for each marker
432 tested. Importantly, untreated PC3 progeny-1 displayed
433 significantly higher expression in all three survival markers tested
434 compared to parental DMSO control cells, suggesting these
435 markers were retained following treatment (Fig. 5A, 5C). CD45 is
436 typically utilized as a tumor cell exclusion marker that stains for
437 white blood cells. At day 10 post-treatment release time points we
438 noted a gain in CD45 protein expression that was retained in
439 progeny cells in PC3 cells (Fig. 5A, S10). MDA-MB-231 cells also
440 showed a significant upregulation of expression for most markers
441 tested, except HOMER1 for docetaxel day 10 post-treatment
442 release and LRP1 for cisplatin day 10 post-treatment release (Fig.
443 5D, 5F).

444
445 **HOMER1, TNFRSF9, and LRP1 are found at the protein level**
446 **patient BM samples, and their increased expression is**
447 **correlated with recurrence in public datasets**

448 A subset of bone marrow samples that displayed a high
449 frequency of CTC-IGC from the prostate cancer patient cohort
450 (Fig. 1) were stained with HOMER1, TNFRSF9, and LRP1 (Fig.

451 6A). Patient 1 and patient 3 did not participate in the clinical trial
 452 but also displayed a high frequency of CTC-IGC (Table 1; see
 453 methods). All patients profiled had CTCs that were positive for the
 454 tested markers (Fig. 6B). While there were CTC-IGC positive for
 455 the marker genes in each patient sample (Fig. 6A), the tested
 456 markers were not selective for CTC-IGC (Fig. S12). Patient-5, who
 457 displayed the highest percentage of CTCs positive for markers
 458 HOMER1 and TNFRSF9, had the shortest PFS at 1.4 months
 459 (Fig. 6B, Table 1). Additionally, these survival markers identified
 460 cells in the bone marrow that displayed increased genomic
 461 content but were negative for canonical epithelial markers (Fig.
 462 S13).

463 In publicly available data for previously treated patients,
 464 high expression of TNFRSF9 and LRP1 significantly correlated
 465 with a shorter progression free survival in patients with prostate
 466 cancer; HOMER1 was not statistically significant (p-value = 0.183)
 467 (Fig. 6C). High gene expression of TNFRSF9, HOMER1, and
 468 LRP1 were all significantly correlated with worse relapse free
 469 survival in breast cancer (Fig. 6D). Taken together, we can
 470 conclude the survival genes are associated with recurrence at the
 471 RNA level and are present on CTCs-IGC in the bone marrow
 472 aspirate of late-stage prostate cancer patients.

473 **4. Discussion**

474 Our analysis of bone marrow liquid biopsy samples from
 475 previously treated advanced prostate cancer patients reveals that

476 the presence of polyploid cancer cells correlates with poorer
477 progression-free survival. Although clinical reports have frequently
478 observed polyploid cancer cells in later disease stages, a direct
479 link with disease recurrence has not been firmly established. We
480 also found that CTC-IGC have copy number profiles identical to
481 typical CTCs and are predominantly present in the bone marrow
482 rather than in peripheral blood.

483 Through single cell copy number profiling and the isolation
484 of progeny from individual polyploid cells, we demonstrate that the
485 polyploid cancer cell phenomenon represents a change in cell
486 state.

487 Single-cell copy number profiling shows that the copy
488 number ratios in patient CTC-IGC as well as chemotherapy
489 induced polyploid MDA-MB-231 and PC3 cells that survive
490 treatment are identical to those in their paired non-polyploid
491 samples. This indicates that these cells, either identified as patient
492 CTCs or those that survive in the days following therapy release *in*
493 *vitro*, undergo multiple rounds of WGD without any additional copy
494 number alterations. These findings provide crucial insights into the
495 dynamics and genetic stability of the polyploid cancer cell state.

496 Obtaining proliferative progeny proved challenging; after
497 three months of culturing single isolated polyploid cells, we
498 successfully derived only one proliferative progeny clone
499 (1/1,440). This outcome is significant for two main reasons: first, it
500 demonstrates that polyploid cancer cells can give rise to progeny,
501 but second, the extremely low success rate underscores why

502 these cells have historically been understudied. To enhance our
503 understanding, future research should employ high-throughput
504 techniques to isolate larger numbers of single cells, such as tens
505 of thousands, which may prove critical in understanding the roles
506 of non-proliferative polyploid cancer cells and assessing their
507 capabilities at reinitiating cell division to give rise to progeny.
508 Additionally, slight variations in the copy number profiles, such as
509 a 3p gain observed in the progeny-1 clone, hint at genomic
510 evolution. Further studies should explore this genomic evolution in
511 different progeny clones once they are sufficiently collected to
512 understand the dynamics of genomic re-organization in these
513 cells.

514 Through *in vitro* single cell transcriptomics, we further
515 provide evidence that polyploid cancer cells display a convergent
516 phenotype between MDA-MB-231 (breast cancer) and PC3
517 (prostate cancer) model systems. Despite being induced with
518 chemotherapies with contrasting mechanisms of action (cisplatin
519 and docetaxel), the different tumor models displayed a shared
520 polyploid signature of upregulating 309 common genes. This
521 convergence reveals significant insights into the biological
522 features of polyploid cancer cells.

523 In our observations, approximately 50% of polyploid cancer
524 cells remained attached to the culture flask in a non-proliferative
525 state during single cell progeny outgrowth experiments. Polyploid
526 cancer cells have been identified to progress through the cell
527 cycle but do not proliferate (i.e., endocycling or cytokinesis failure

528 occur before mitosis) [55]. This is hypothesized to be a protective
 529 state of the cells that affords protection from therapeutic stressors.
 530 This phenomenon aligns with our identification of ZNF697 and
 531 NPAS2 as two transcription factors significantly enriched in the
 532 convergent polyploid gene set that were previously identified to be
 533 upregulated in cells that were in a non-proliferative state and
 534 began re-initiating cell division [47]. This suggests that some
 535 polyploid cells profiled on day 10 post therapy release may be
 536 attempting to re-initiate proliferation since the chemotherapy has
 537 been removed. This finding is further supported by a higher
 538 percentage of cells at 10-DPT expressing more markers at the M-
 539 phase of the cell cycle (Fig. S8-9). Future research should explore
 540 the roles of ZNF697 and NPAS2 in polyploid cancer cells and their
 541 implications for disease recurrence in progeny cells.

542 The convergent surviving cell gene set we identified
 543 indicated that pro-survival and anti-apoptotic pathways, such as
 544 TNFa via NFkB, PI3K-AKT, and mTOR signaling, are upregulated
 545 in polyploid cancer cells [57-59]. Among the genes identified in
 546 these pathways, TNFRSF9, HOMER1, and LRP1 were identified
 547 as putative survival genes and were found to be upregulated at
 548 the RNA and protein levels [48-54, 60-64]. Notably, these protein
 549 markers were retained in the PC3 progeny-1 clone, suggesting
 550 their upregulation in cells that survive chemotherapy. Additionally,
 551 a subset of CTCs, including both polyploid and typical CTCs,
 552 tested positive for TNFRSF9, HOMER1, and LRP1 at the protein
 553 level. Of note, Patient 5, who experienced the shortest

554 progression-free survival at 1.4 months, had the highest
555 percentage of CTCs positive for the TNFRSF9 marker, indicating
556 that this gene may play a significant role in cancer cell survival.
557 Further, these markers identified a subset of cells with IGC that
558 were negative in the epithelial channel. These cells may be CTCs
559 that lost epithelial expression (i.e., EMT) and, in combination with
560 the upregulation of the proposed survival markers, could be adept
561 at surviving in the bone marrow. Further studies are needed to
562 evaluate the roles of TNFRSF9, HOMER1, and LRP1 in
563 chemotherapy resistance and as a biomarker to evaluate the
564 emergence of therapeutic resistance.

565 Our investigation of polyploid cancer cells confirms the
566 significant upregulation of hypoxia and cholesterol homeostasis
567 pathways. Studies have shown that targeting these pathways in
568 cell line models, including PC3 and MDA-MB-231, reduces the
569 viability of progeny from polyploid cancer cells [23,26]. Further
570 evidence comes from a study indicating that polyploid cancer cells
571 accumulate lipid droplets in response to chemotherapy [56],
572 underscoring the critical role of lipid balance as cells significantly
573 increase in size. These findings suggest that these pathways are
574 integral to the polyploid cancer cell state and represent promising
575 targets for therapeutic intervention.

576 The *in vitro* environment of cell culture does not always
577 recapitulate the *in vivo* nature of cancer cell biology. This makes it
578 difficult to speculate how polyploid cancer cells interact with their
579 neighboring malignant cells and the surrounding stroma.

580 Translating the findings of TNFRSF9, HOMER1, and LRP1 as
 581 resistance markers in an *in vivo* model is a critical next step.
 582 Future studies should employ mouse models or patient derived
 583 xenografts and stain for these biomarkers to understand their
 584 prominence *in vivo*. Further studies should also isolate polyploid
 585 cancer cells through nuclear density to further understand their
 586 cellular phenotypes in tumor tissue.

587 While patient results are promising, they also have
 588 limitations. This study focuses on late-stage patients with
 589 disseminated CTCs in the bone marrow and blood. The evaluated
 590 cohort comprised advanced-stage patients whose previous
 591 treatment regimens had failed. To minimize biases associated with
 592 late-stage disease and to better understand initial treatment
 593 responses and their role in inducing polyploid cancer cells, future
 594 cohorts should include patients undergoing their first rounds of
 595 therapy. One concern is that CTCs in peripheral blood are typically
 596 found in later disease stages, potentially biasing our patient
 597 population towards later stages. Obtaining samples from tissue,
 598 blood, and bone marrow could address these concerns and
 599 provide valuable insights into the role of polyploid cancer cells in
 600 dissemination, initial response to therapy, and disease evolution.

601

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626 involved in the study.

627 **Data Availability:** Cell line scDNA-seq (GSE270567) and scRNA-
628 seq (GSE270568) are available through GEO. Patient scDNA-seq
629 is available upon reasonable request. Image data is available
630 upon reasonable request for cell lines and patients.

631 **Materials Availability:** If interested in using the High Definition
632 Single Cell Assay please contact CSI-Cancer.
633 **Code Availability:** Image analysis code is freely available at
634 <https://github.com/aminnaghdloo/slide-image-utils>. Downstream
635 analysis scripts (DNA-seq, RNA-seq, image quantification) are
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637 **Author Contribution:** Conceptualization: M.J.S., R.K.P., K.J.P.,
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649

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Figure legends

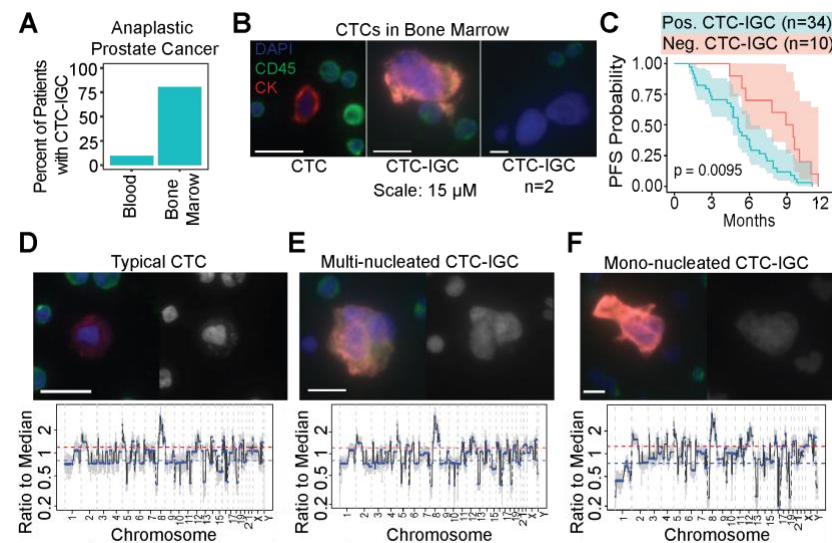


Figure 1: Large tumor cells are found in BM of late-stage prostate cancer patients. (A) Enumeration of patients with matched blood and bone marrow samples with at least 1 CTC-IGC present in liquid biopsy. (B) Representative images of CTC and CTC-IGC found in BM aspirate. Scale bars set to 15 μ M. (C) PFS from patients with or without CTC-IGC found in BM samples. (D) Representative image of typical CTC found in BM with merged and DAPI channels (top) and its genomic copy number profile (bottom). (E) Representative image of CTC-IGC found in bone marrow with merged and DAPI images (top) and its genomic copy number profile (bottom). (F) Representative image of mono-nucleated CTC-IGC found in bone marrow with merged and DAPI images (top) and its genomic copy number profile (bottom).

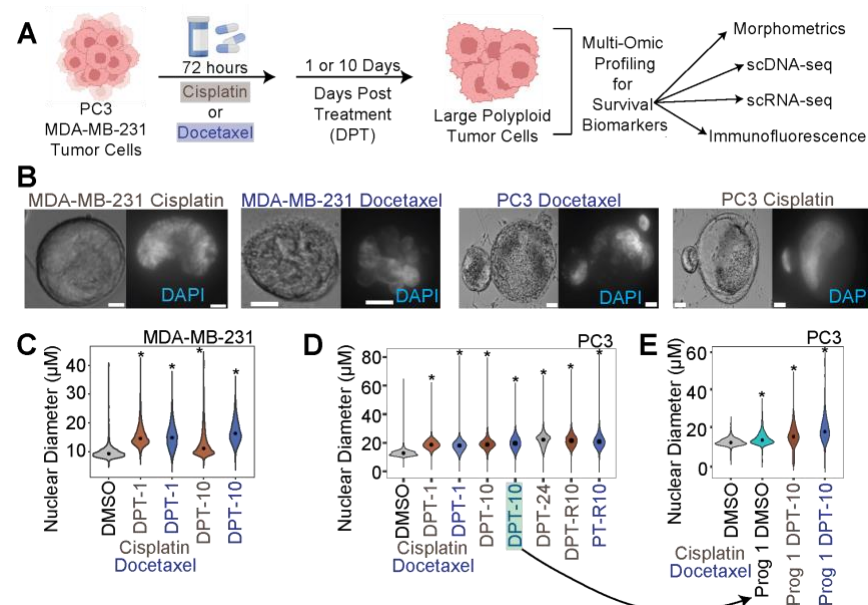


Figure 2: Large polyploid tumor cells are induced following chemotherapy exposure in MDA-MB-231 and PC3 cell lineages. (A) Experimental schematic for in vitro investigation of surviving polyploid cells. (B) Representative bright field (left) and DAPI (right) 40x images of MDA-MB-231 cells and PC3 cells treated with docetaxel and cisplatin (right) 10 days post treatment recovery. (C) Nuclear diameter for all treated conditions for MDA-MB-231 cells. (D) Nuclear diameter for all treated conditions for PC3 cells. Arrow indicates the condition where single cell progeny originated from. (E) Nuclear diameter of PC3 control parental and progeny-1 cells, and progeny-1 10 days post-treatment cells.

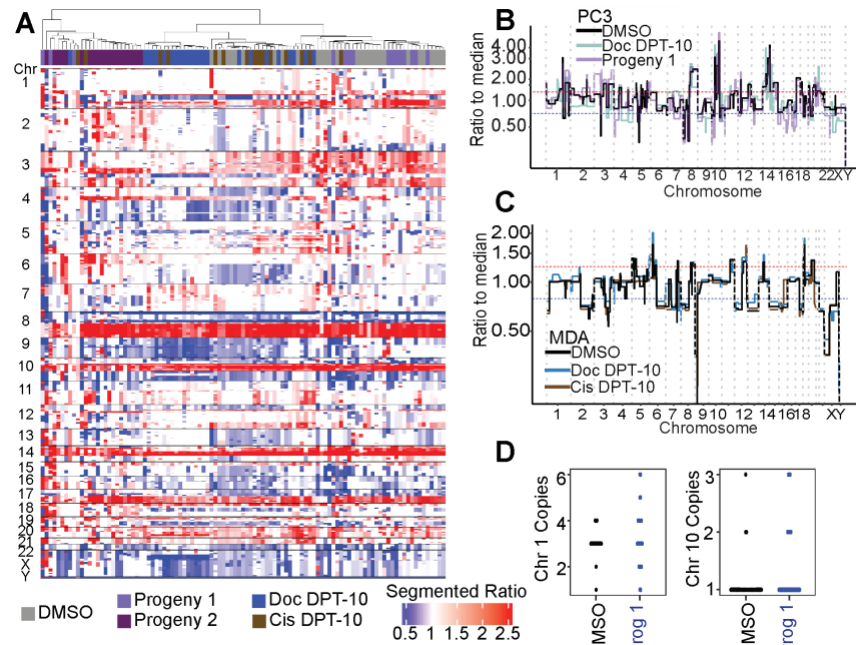


Figure 3: Genomics of PC3 DMSO control, docetaxel treated cells, and docetaxel large cell progeny-1. (A) Segmented copy number ratios for PC3 conditions. Ratio of 1 (white) indicates copy number neutral respective to the entire genome. (B) Representative PC3 DMSO, Doc D10, and Progeny-1 copy number ratio profiles. (C) Representative MDA-MB-231 DMSO control, Doc D10, and Cis D10 copy number ratio profiles. (D) FISH for PC3 DMSO control and progeny-1 cells for centromere of chromosome 1 (ploidy = 3) and chromosome 10 (ploidy = 1).

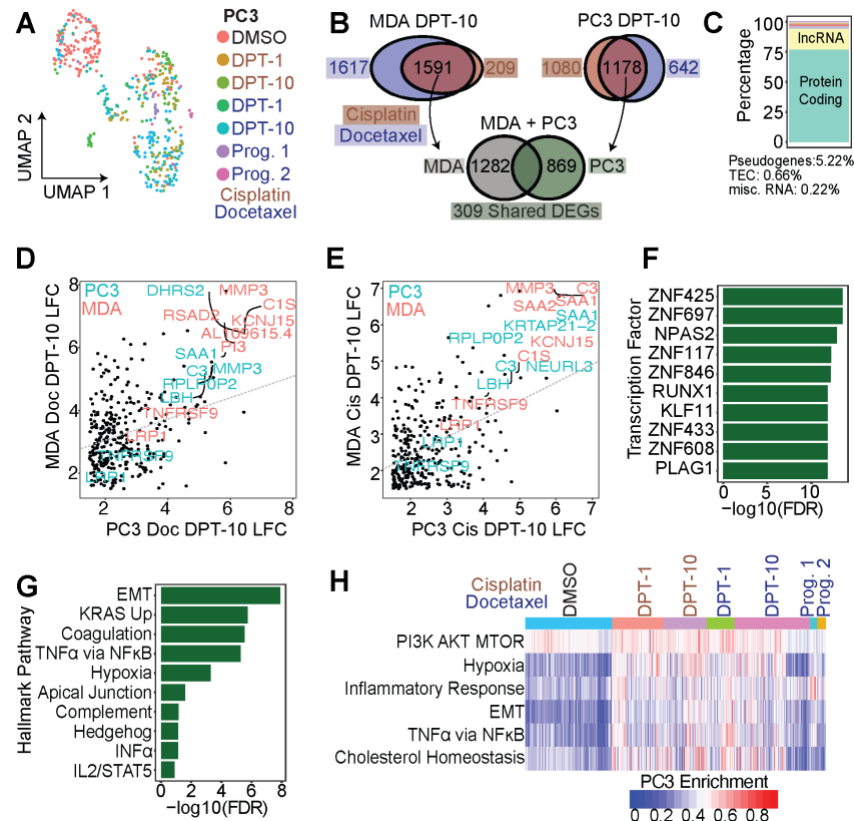


Figure 4: Chemotherapy induced surviving tumor cells share common phenotypes and pathways for survival. (A) UMAP of all conditions for PC3 cells. (B) Comparison of DEGs between MDA-MB-231 large-D10 and PC3 large-D10 cells. Between cisplatin and docetaxel treatments, MDA-MB-231 and PC3 share 309 upregulated genes compared to their respective controls. (B) Genecode annotations for 309 shared genes. (D-E) LFC of shared 309 genes for PC3 vs MDA-MB-231 for docetaxel and cisplatin treatments, respectively. (F) CHEA3 transcription factor enrichment of the shared 309 genes between MDA-MB-231 and PC3 cells. (G) Hallmark enrichment analysis of 309 shared genes. (H) Single cell Hallmark gene set enrichment analysis for all PC3 cells.

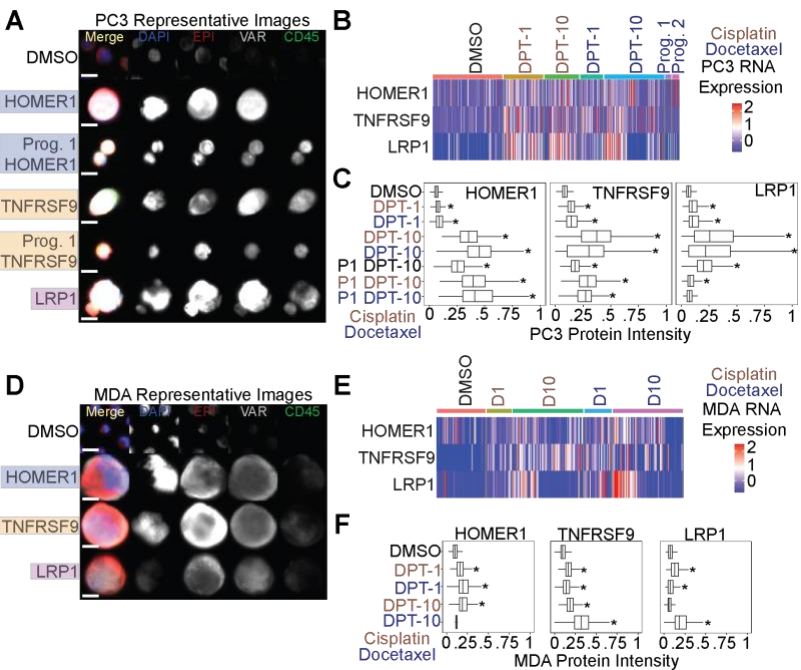


Figure 5: HOMER1, TNFRSF9, and LRP1 are putative markers of chemotherapy resistance. (A) Representative PC3 images of putative marker genes stain in the VAR (4th) channel. DMSO control cells were stained with HOMER1 and were negative. (B) RNA expression for each marker for all PC3 cells. (C) Immunofluorescence quantification for PC3 cells stained with tested markers. (D) Representative MDA-MB-231 images of putative marker genes stained in the VAR (4th) channel. DMSO control cells were stained with HOMER1 and were negative. (E) RNA expression for each marker for all MDA-MB-231 cells. (F) Immunofluorescence quantification for MDA-MB-231 cells stained with tested markers.

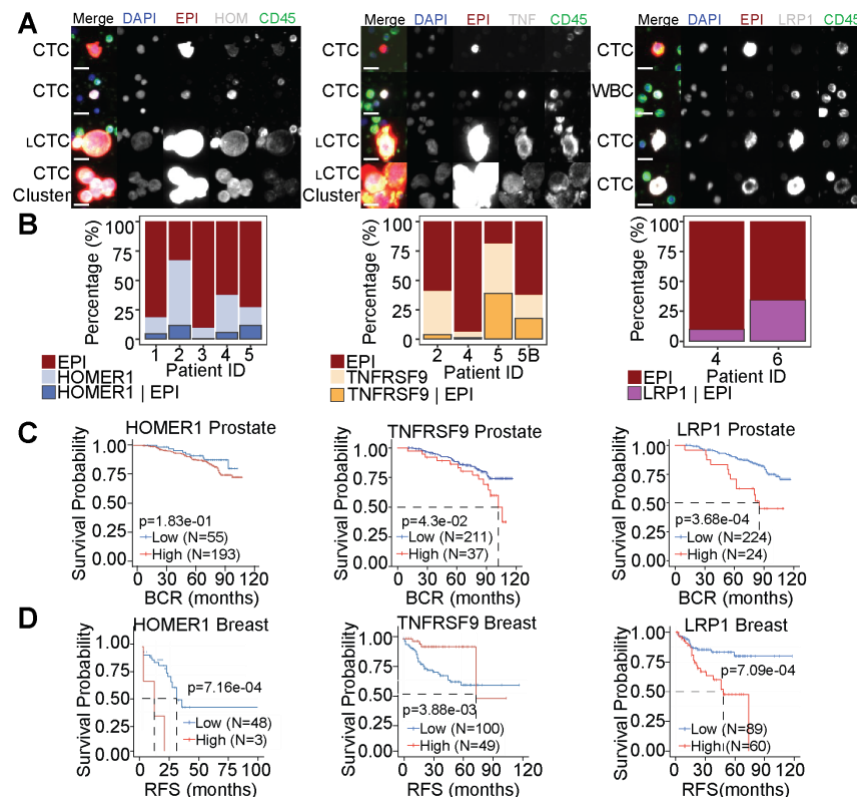


Figure 6: HOMER1, TNFRSF9, and LRP1 are positive on CTCs in the BM aspirate of late-stage prostate cancer and are correlated with recurrence in prostate and breast cancers. (A) Representative CTCs from BM of advanced prostate cancer patients that were stained with survival markers HOMER1 (left), TNFRSF9 (TNF; middle), and LRP1 (right). Tested markers appear as white in the merged image. Scale bars are 15 μ M. (B) Percentages of CTCs with EPI positivity and cells that were stained with survival markers HOMER1 (left), TNFRSF9 (middle), and LRP1 (right). Cells that are positive for the marker alone (middle bar) cannot be conclusively labeled a tumor derived cell. LRP1 (right) is also a marker of T-cells, so only cells that were EPI positive were included. (C-D) Kaplan-Meier survival plots for RNA expression of tested markers in prostate (C) and (D) breast cancer patients.