RUNX1 mitotically bookmarks target genes that are important for the mammary epithelial-to-mesenchymal transition

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27 **Significance:**

28 This study elucidates mitotic gene bookmarking as a potential epigenetic
29 mechanism that impacts breast epithelial cell growth and phenotype and has potential
30 implications in breast cancer onset.

ABSTRACT

RUNX1 has recently been shown to play an important role in determination of mammary epithelial cell identity. However, mechanisms by which loss of the RUNX1 transcription factor in mammary epithelial cells leads to epithelial-to-mesenchymal transition (EMT) are not known. Here, we report mitotic bookmarking of genes by RUNX1 as a potential mechanism to convey regulatory information through successive cell divisions for coordinate control of mammary cell proliferation, growth, and identity. Genome-wide RUNX1 occupancy profiles for asynchronous, mitotically enriched, and early G1 breast epithelial cells reveal RUNX1 is retained during the mitosis to G1 transition on protein coding and long non-coding RNA genes critical for mammary epithelial proliferation, growth, and phenotype maintenance. Disruption of RUNX1 DNA binding and association with mitotic chromosomes alters cell morphology, global protein synthesis, and phenotype-related gene expression. Together, these findings show for the first time that RUNX1 bookmarks a subset of epithelial-related genes during mitosis that remain occupied as cells enter the next cell cycle. Compromising RUNX1 DNA binding initiates EMT, an essential first step in the onset of breast cancer.

INTRODUCTION

Breast cancer arises from a series of acquired mutations and epigenetic changes that disrupt normal mammary epithelial homeostasis and create multi-potent cells that can differentiate into biologically unique and clinically distinct subtypes (1-6). Epithelial-to-mesenchymal transition (EMT)—a trans-differentiation process through which mammary epithelial cells acquire the aggressive mesenchymal phenotype—is a key driver of breast cancer progression, invasion and metastasis (7-12). Transcription factors Snail, Slug, Twist, and Zeb1/2 contribute to EMT during early, normal development and have also been implicated in invasion (13-16). Despite accumulating evidence that defines a broad understanding of EMT regulation and maintenance of the epithelial phenotype (7-12), the mechanism(s) by which mammary cells maintain their epithelial phenotype is unknown.

Runt-Related Transcription Factor 1 (RUNX1/AML1) is required for hematopoietic lineage specification during development and hematopoiesis throughout life (17-30). In addition to the recognized role in hematological malignancies, RUNX1 has been recently identified as a key player in breast cancer development and tumor progression (31-38). Findings from our group (39), reinforced by studies from others (40, 41), have shown that RUNX1 plays a critical role in maintaining breast epithelial phenotype and prevents EMT through transcriptional regulation of genes (e.g., the EMT marker and a key cell adhesion protein E-cadherin) involved in fundamental cellular pathways. However, mechanisms by which RUNX1 prevents EMT have not been identified.

Mitotic gene bookmarking, i.e. transcription factor binding to target genes during mitosis for transcriptional regulation following cell division, is a key epigenetic mechanism

71 to convey and sustain regulatory information for cell proliferation, growth, and cell identity
72 from parent to progeny cells (42-49). We have established that RUNX proteins, as well
73 as other phenotypic transcription factors that include MYOD and CEBPα, mitotically
74 bookmark RNA Pol I- and II-transcribed genes in osteoblasts and leukemia cells for
75 coordinate control of cell growth, proliferation and phenotype (50-57). It is increasingly
76 evident that mitotic gene bookmarking by transcription factors is a key mechanism to
77 determine and maintain cell fate across successive cell divisions (58-70).

78 We addressed the hypothesis that RUNX1 maintains the breast epithelial
79 phenotype by mitotic bookmarking of genes that support mammary epithelial proliferation,
80 growth, and phenotype for expression in the next cell cycle. Fluorescence confocal
81 microscopy of fixed and live mammary epithelial cells revealed that RUNX1 is present on
82 chromosomes throughout mitosis and colocalizes with upstream binding transcription
83 factor (UBF), a subunit of RNA Pol I transcriptional machinery (71). To identify genes
84 occupied by RUNX1, we performed chromatin immunoprecipitation coupled with high
85 throughput sequencing (ChIP-Seq) using a RUNX1-specific antibody on mitotic, G1, and
86 asynchronous normal mammary epithelial MCF10A cells. As expected, ribosomal RNA
87 genes, regulated by the RNA Pol I transcriptional machinery, were occupied by RUNX1.
88 A fluorescence-based, global protein synthesis assay showed reduced protein synthesis
89 when RUNX1 DNA binding was perturbed using a small molecule inhibitor. Importantly,
90 ChIP-Seq revealed that, in mitosis, RUNX1 associates with RNA Pol II regulated genes
91 specifically involved in maintenance of the epithelial phenotype and EMT progression.
92 Strikingly, disruption of RUNX1 DNA binding, which is required for association with mitotic
93 chromosomes (56), results in loss of the epithelial phenotype and acquisition of

94 mesenchymal properties that are accompanied by changes in expression of associated
95 genes and pathways and represent early events in the onset of breast cancer. These
96 findings implicate RUNX1 occupancy of target genes at the mitosis into G1 transition in
97 regulating the normal breast epithelial phenotype.
98

99 **RESULTS**

100 **RUNX1 associates with mitotic chromatin and occupies target genes**

101 To investigate subcellular localization of RUNX1 in normal mammary epithelial
102 cells, we performed immunofluorescence (IF) microscopy in actively proliferating
103 MCF10A cells and imaged cells undergoing spontaneous mitoses. We observed that
104 RUNX1 is localized on mitotic chromatin at all topologically identified substages of mitosis
105 (Fig. 1; top panels). Two distinct types of foci are detectable on mitotic chromosomes: 2-
106 8 large punctate foci that appear to be allelic as well as numerous smaller foci that are
107 distributed across the chromosomes (Fig. 1; bottom panels, white arrowheads). In all
108 replicates, important secondary-antibody-only controls were included to confirm
109 specificity of RUNX1 signal on mitotic chromosomes (Suppl. Fig. 1). In the interphase
110 nuclei, RUNX1 exhibited the characteristic punctate distribution (Fig. 1; interphase panel).
111 To ensure reproducibility of our findings, the IF experiments were repeated at least 3
112 times and, at the minimum, 20 interphase and mitotic cells were imaged.

113 Multiple reports have indicated that formaldehyde fixation can prevent regulatory
114 protein detection on mitotic chromosomes (58, 69). To further confirm that association of
115 RUNX1 with mitotic chromosomes is not under-represented because of formaldehyde
116 fixation, we examined the localization of RUNX1-EGFP in actively proliferating, unfixed
117 MCF10A cells. Consistent with our findings in fixed and synchronized cells, RUNX1-
118 EGFP was associated with chromosomes in live MCF10A cells undergoing mitosis
119 (Suppl. Fig. 2.). Together, these findings establish that RUNX1 foci are present on
120 chromosomes at all stages of mitosis under physiological conditions in actively dividing,

121 unfixed breast epithelial cells and, in agreement with our previous findings, are equally
122 distributed into resulting progeny cells (57).

123 To experimentally address whether RUNX1 presence on mitotic chromosomes
124 reflects occupancy of target genes, MCF10A cells were synchronized in mitosis using
125 nocodazole (50ng/mL; Supp. Fig. 3A). Nocodazole dose and treatment were empirically
126 determined to minimize toxic effects of the drug, while maximizing mitotic enrichment.
127 Mitotic cells were collected by mitotic shake-off and purity of harvested cells was
128 confirmed by the presence of H3pS28 in >70% of cells. We chose the H3pS28 mark to
129 identify mitotic cells because this histone mark is highly specific to condensed
130 chromosomes during mitosis; more commonly used H3pS10 mark is additionally
131 observed in early G1 cells and has also been associated with replicating centers in S-
132 phase (72, 73). A parallel, nocodazole-treated cell population was released into early G1
133 by replacing nocodazole-containing growth medium with fresh, nocodazole-free, growth
134 medium and was harvested 3 hours post-release (Suppl. Fig. 3B). Western blot analysis
135 of whole cell lysates from the three cell populations showed expected levels of expression
136 for cell cycle-specific proteins Cyclin B and CDT1 (Suppl. Fig. 3C). FACS profiles of the
137 cell populations confirmed the characteristic enrichment of blocked cells in mitosis (Supp.
138 Fig. 3D; Mitotic) and release into G1 upon media replacement (Supp. Fig. 3D; G1) when
139 compared to asynchronous cells (Supp. Fig. 3D; Asynch). To determine whether RUNX1
140 remains bound to target genes during mitosis, ChIP-Seq was performed on Asynch,
141 Mitotic, and G1 MCF10A cells using a RUNX1 specific antibody (Fig. 2A). Enriched
142 regions (peaks called by MACS2) were compared using k-means clustering (k=4) of
143 normalized enrichment profiles of the three cell populations. This analysis revealed

144 subsets of genes that were either shared across the three groups or were specific for
145 each, indicating dynamic binding of RUNX1 during and immediately after mitosis (Fig.
146 2A). Peak calling identified RUNX1 occupancy of both protein coding and long non-coding
147 RNA (lncRNA) genes. Specifically, RUNX1 occupied 1070 genes in cell population not in
148 G1 or M phase (Fig. 2A; green bar) and 1095 genes in G1-enriched cells (Fig. 2A; light
149 red bar). Importantly, RUNX1 occupied 551 genes (413 protein coding and 138 lncRNAs)
150 in mitotically enriched MCF10A cells (Fig. 2A; blue bar).

151 Functional relevance of RUNX1 occupancy in the three cell populations was
152 determined by comparing RUNX1-occupied genes with those that are differentially
153 regulated upon shRNA-mediated RUNX1 knockdown (39). Of the 1070 genes occupied
154 by RUNX1 in cell populations not in G1 or M phases, 353 genes were deregulated upon
155 RUNX1 depletion (Fig. 2A). Importantly, RUNX1 depletion deregulated 399 of 1268
156 RUNX1-bound genes in the M and G1 populations. These findings reveal that several
157 hundred target genes are bookmarked by RUNX1 during mitosis and transcriptionally
158 regulated in normal mammary epithelial cells.

159 To identify cellular processes and pathways that are comprised of RUNX1-
160 bookmarked genes, we performed gene set enrichment analysis (GSEA) on genes bound
161 by RUNX1 during mitosis or G1, or not bound in either cell cycle stage (Fig. 2B).
162 Interestingly, most genes bookmarked by RUNX1 during mitosis were associated with
163 negative regulation of gene expression and metabolic process (Fig. 2B; blue box).
164 Consistent with a cellular requirement to reattach and enter the next cell cycle and fully
165 resume transcription, genes bound during G1 were primarily enriched in biological
166 processes involving cell anchorage, protein localization and positive regulation of gene

167 expression (Fig. 2B; red box). ChIP-seq results were further validated by motif analysis
168 of RUNX1-bound peaks, which showed that the RUNX motif was the most enriched motif
169 in all three cell populations (Fig. 2C). Importantly, RUNX1-bound genomic regions were
170 also enriched in motifs for transcription factors (e.g., Fra1, JunB, ETS) known to
171 cooperate with RUNX1 for gene regulation (74)(Fig. 2C). Together, these findings indicate
172 that RUNX1 occupies genes involved in cell proliferation, growth, and phenotype during
173 mitosis in normal mammary epithelial cells.
174

175 **RUNX1 mitotically bookmarks RNA Pol I-transcribed genes that control cell growth**

176 Our ChIP-Seq results revealed that RUNX1 occupies rDNA repeats in MCF10A
177 mammary epithelial cells; all three MCF10A cell populations (Asynch, Mitotic and G1)
178 exhibited significant fold enrichment within the promoter region of hrDNA (Fig. 3A),
179 suggesting a potential role for RUNX1 in regulating rRNA genes in MCF10A cells. We
180 confirmed this finding in actively proliferating MCF10A cells by immunofluorescence
181 microscopy for antibodies specific against RUNX1 and upstream binding factor (UBF), a
182 transcriptional activator that remains bound to rRNA genes during mitosis (75). We
183 observed large RUNX1 foci colocalizing with UBF throughout each stage of mitosis (Fig
184 3B and Suppl. Fig. 4). Colocalization between RUNX1 and UBF was validated by confocal
185 microscopy. Line scans of MCF10A cells show that although RUNX1 and UBF occupy
186 distinct nuclear microenvironments in interphase (n=15), both proteins substantially
187 colocalize in metaphase (n=15)(Suppl. Fig. 4). Taken together, these findings establish
188 RUNX1 binding to ribosomal DNA repeat regions by ChIP-Seq (Fig. 3A) and confirmed
189 at the cellular level by confocal microscopy (Fig 3B & Suppl. Fig. 4).

190 We experimentally addressed the hypothesis that RUNX1 regulates ribosomal
191 RNA gene expression by using a pharmacological inhibitor of RUNX1. The small
192 molecule inhibitor AI-14-91, which has been extensively characterized by several groups,
193 interferes with RUNX1-CBFβ interaction and disrupts RUNX1 DNA binding (76, 77). We
194 examined the effect of RUNX1 inhibitor on pre-rRNA expression and found that pre-rRNA
195 expression was significantly increased at 12hr and 48hr time points after treatment of
196 asynchronous cells with the specific RUNX1 inhibitor but not the control inactive
197 compound AI-4-88, indicating that RUNX1 suppresses rRNA gene expression in normal
198 mammary epithelial cells (Fig. 3C). Because levels of rRNA directly correlate with global
199 protein synthesis, a fluorescent-based detection method was used to measure newly
200 synthesized proteins. Cells treated with AI-14-91 for 24hr or 48hr showed a moderate
201 change in levels of global protein synthesis in comparison to AI-4-88 control-treated cells
202 under identical conditions (n=3; Fig. 3D). Together, our results demonstrate that RUNX1
203 bookmarks RNA Pol I regulated rRNA genes during mitosis and transcriptionally
204 represses them during interphase with moderate impact on global protein synthesis in
205 normal mammary epithelial cells.
206

207 **RUNX1 occupies RNA Pol II-transcribed genes involved in hormone-**
208 **responsiveness and cell phenotype during mitosis**

209 Using RUNX1-bookmarked genes, gene set enrichment analysis (GSEA) was
210 performed to identify regulatory pathways (Fig. 4A). In agreement with known roles of
211 RUNX1 (78-82), the top 10 pathways identified were those involved in regulation of G2M
212 Checkpoint, E2F targets, p53, and DNA repair (Fig. 4A). Consistent with our finding that

213 RUNX1 bookmarks and regulates rRNA genes, one of the pathways identified is mTOR
214 signaling, a pathway that is required for cell growth and is a therapeutic target in breast
215 cancers (83, 84). Relevant to the normal mammary epithelial phenotype, both early and
216 late estrogen responsive gene sets significantly overlap with RUNX1 mitotically
217 bookmarked genes (Fig. 4A). Because estrogen plays vital roles in promoting proliferative
218 phenotypes of mammary epithelial cells (85-87), we interrogated RUNX1 bookmarked
219 genes to identify those bound by RUNX1 and ERα in MCF7 cells, where RUNX1
220 contributes to higher order genome organization (Fig. 4B) (88, 89). Using publicly
221 available datasets of ERα genome-wide occupancy and estradiol-regulated gene
222 expression (GSE40129)(90), we find that a subset of genes mitotically bookmarked by
223 RUNX1 is also bound by ERα, and either up or down regulated in response to estradiol.
224 These findings indicate that RUNX1-bookmarked genes are involved in pathways that
225 control hormone-responsiveness, proliferation and growth of normal mammary epithelial
226 cells (Fig. 4B).

227 We identified a novel subset of genes that are bookmarked by RUNX1 and relate
228 to regulatory pathways involved in cellular phenotype including TNFα, Apical Junction
229 and Notch signaling (Fig. 4A). Furthermore, NEAT1 and MALAT1, lncRNAs often
230 deregulated in breast cancer (91, 92), were also mitotically bookmarked by RUNX1. Of
231 the 413 RUNX1 bookmarked protein coding genes, *TOP2A*, *MYC*, *HES1*, *RRAS*, *H2AFX*,
232 and *CCND3* are representative of RNA Pol II-transcribed genes involved in phenotype
233 maintenance and cell fate decisions (See Suppl. Table 1 for complete list). Recently,
234 *HES1* and *H2AFX* have been identified as regulators of breast epithelial phenotype (93-
235 95). In our ChIP-seq dataset, *HES1* and *H2AFX* show significant fold enrichment of

236 RUNX1 occupancy between the three populations of MCF10A cells (Fig. 4C; top panels).
237 Expression of *HES1* increased upon inhibition of RUNX1 DNA binding (Fig. 4C; left
238 panel—bar graph), indicating that RUNX1 represses *HES1*. In contrast, *H2AFX*
239 expression at 24hr and 48hr of inhibitor treatment was decreased, suggesting RUNX1
240 activates H2AFX expression (Fig. 4C; right panel—bar graph). These results suggest that
241 RUNX1 bookmarks both protein coding and non-coding genes that are critical
242 determinants of epithelial lineage identity as a potential mechanism to stabilize the
243 mammary epithelial phenotype.
244

245 **Inhibition of RUNX1 DNA binding causes epithelial to mesenchymal transition and**
246 **alters the associated transcriptome**

247 To experimentally address whether disruption of RUNX1 bookmarking leads to a
248 change in epithelial phenotype, we treated cells with RUNX1 DNA binding inhibitor and
249 the inactive control compound and monitored changes in cell morphology (Fig. 5A).
250 Consistent with RUNX1 bookmarking and regulation of genes critical for epithelial
251 phenotype (Figs. 2 & 4), disruption of RUNX1 DNA binding for 48 hours resulted in
252 mesenchymal morphology. We next examined whether long-term inhibition of RUNX1
253 caused a permanent change in cell phenotype. Longer term treatment (5 days) of actively
254 proliferating MCF10A cells showed significant apoptosis, although a small sub-population
255 of cells survived and exhibited an altered phenotype (Fig. 5B). The surviving sub-
256 population at day 5 was recovered by culturing cells in media without the inhibitor. By day
257 3-4 following media replacement, cells clearly showed a mesenchymal morphology (Fig.
258 5B), indicating that interfering with RUNX1 DNA binding causes loss of the normal

259 mammary epithelial phenotype. Consistent with changes in cell morphology, we find
260 alterations in expression and localization of the cytoskeletal F-actin protein (Fig. 5C).
261 These observations were confirmed by examining the expression of epithelial markers
262 (e.g., E-cadherin (Fig. 5D)), as well as mesenchymal markers (e.g., Vimentin (Fig. 5D)).
263 E-cadherin was largely unchanged; however, Vimentin expression was significantly
264 increased, confirming an epithelial-to-mesenchymal transition upon inhibition of the
265 RUNX1-CBFβ interaction.

266 To identify transcriptome-wide changes associated with EMT upon inhibition of
267 RUNX1 DNA binding activity that is required for retention of the protein with mitotic
268 chromosomes (56), we performed RNA sequencing (in triplicates) at each of the indicated
269 time points (Day 1 and 2 in Crisis Phase and Day 4 and 7 in Recovery Phase). Heatmap
270 analysis of all time points identified substantial changes in gene expression as cells
271 transition from epithelial to mesenchymal phenotype (Fig. 6A). A differential gene
272 expression analysis between the crisis and recovery phases uncovered significant
273 changes in expression of genes associated with EMT (e.g., IL32, SERPINB2 etc.; Fig.
274 6B). Importantly, a subset of differentially expressed genes was occupied by RUNX1
275 during mitosis. We performed pathway analysis on differentially expressed genes (Fig.
276 6C and Suppl. Table 2). Consistent with phenotypic changes, we found that multiple
277 signaling pathways that include TNF alpha, Interferon Gamma and estrogen
278 responsiveness were altered during EMT caused by RUNX1 inhibition.

279 We next determined the effect of the inhibition of RUNX1-CBFβ interaction on
280 mitotic retention of the protein. Actively proliferating MCF10A cells were treated with the
281 inhibitor (AI-14-91) for 6hr, 12hr, 24hr, and 48hr at 20μM; a structurally similar but inactive

282 compound (AI-4-88) was used as a control under identical conditions. Cells were
283 subjected to immunofluorescence microscopy followed by detection of RUNX1 and UBF
284 as described above. Although RUNX1 signal was detected in all mitotic sub-stages
285 (Suppl. Fig. 5), we observed a substantial decrease in RUNX1 signal intensity on
286 metaphase chromosomes (white arrows; Fig. 6D), indicating that RUNX1-CBFβ
287 interaction and RUNX1 DNA binding activity play a key role in mitotic gene bookmarking.
288 These changes were more pronounced for smaller RUNX1 foci and were not observed in
289 control-treated cells; appreciable signal for large RUNX1 foci that colocalize with UBF
290 (Fig. 3B & Suppl. Fig. 4) remained detectable in all sub-stages of mitosis (Fig. 6D and
291 Suppl. Fig. 5). Together, these findings indicate that RUNX1 mitotic bookmarking of
292 genes related to epithelial cell growth, proliferation, and lineage is associated with
293 epithelial cell identity, and disruption of RUNX1 DNA binding leads to mesenchymal
294 transition.

295 **DISCUSSION**

296 This study identifies retention of RUNX1 on mitotic chromosomes and occupancy
297 of target genes as a potential epigenetic mechanism for coordinate regulation of RNA Pol
298 I- and II-transcribed genes that are critical for mammary epithelial proliferation, growth,
299 and phenotype maintenance. Pharmacological inhibition of RUNX1 DNA binding causes
300 transition of mammary epithelial cells to a mesenchymal phenotype, indicating that
301 RUNX1 bookmarking of target genes contributes to stabilizing the normal breast epithelial
302 phenotype.

303 Our findings are the first to examine RUNX1 bookmarking of target genes during
304 mitosis in mammary epithelial cells and to report that RUNX1 coordinately controls cell
305 growth-related ribosomal RNA (rRNA) genes and a large subset of cell
306 proliferation/phenotype-related genes in these cells. One target gene of interest is hairy
307 and enhancer of split-1 (*HES1*). Hes1 is a transcription factor that represses genes
308 involved in cellular development and is regulated primarily by NOTCH signaling, one of
309 our top ten overlapping hallmark gene sets bookmarked by RUNX1 (Fig. 4)(96, 97). HES1
310 was recently shown to have a prominent role in proliferation and invasion of breast cancer
311 cells, and its silencing led to downregulation of p-Akt signaling and ultimately prevented
312 EMT (93). Our findings indicate that RUNX1 stabilizes the normal mammary epithelial
313 phenotype, in part, by bookmarking *HES1* and suppressing its expression.

314 Another important RNA Pol II-transcribed gene mitotically bookmarked by RUNX1
315 and critical for maintaining cellular phenotype is histone variant H2AFX (*H2AFX*).
316 Silencing *H2AFX* in breast epithelial cells leads to induction of EMT through activation of
317 *SNAIL2/SLUG* and *TWIST1* (95). Upon inhibition of the RUNX1-CBFβ interaction, we find

318 a decrease in *H2AFX* expression and a concomitant, significant increase in
319 SNAIL2/SLUG expression. These data identify RUNX1 as a novel upstream regulator of
320 *H2AFX* expression; RUNX1 bookmarking and activation of H2AFX and subsequent
321 suppression of *SNAIL2/SLUG* prevents EMT in breast epithelial cells.

322 Several groups have shown that RUNX1 interacts with ERα at both enhancer
323 regions and transcriptional start sites (TSSs) for regulation of specific genes (34, 88). Our
324 ChIP-Seq results, coupled with publicly available data sets, reveal a novel finding: RUNX1
325 bookmarking of a subset of ERα-occupied, hormone-responsive genes during mitosis
326 may be critical for maintenance of the breast epithelial phenotype. Future studies will be
327 required to investigate mechanistic significance of this observation in estrogen receptor
328 positive mammary epithelial and breast cancer cells.

329 Our findings suggest that mitotic gene bookmarking by RUNX1 contributes to
330 regulation of the mammary epithelial phenotype. Equally important, our study shows that
331 inhibition of RUNX1 DNA binding specifically elicits an epithelial-to-mesenchymal
332 transition that accompanies changes in critical genes and pathways involved in EMT.
333 These findings are further supported by RUNX1 mitotic occupancy of cell growth-related
334 rRNA genes, and together highlight a key role of RUNX1 in coordinating cell proliferation,
335 growth and phenotype. Because RUNX1 interacts with multiple co-activators and co-
336 repressors, additional in-depth studies are required to determine contributions of RUNX1
337 co-regulatory proteins to mitotic gene bookmarking.

338 Another novel contribution of the current study is mitotic bookmarking of lncRNAs
339 by a transcription factor. RUNX1 was recently shown to regulate lncRNAs NEAT1 and
340 MALAT1 (89, 91), which have critical roles in the onset and progression of breast cancer

341 (92). Our findings show that, in addition to occupying protein coding genes, RUNX1
342 bookmarks several lncRNAs for post-mitotic regulation. It will be important to determine
343 if RUNX1-bookmarked lncRNAs have G1-specific roles in maintaining the normal
344 mammary epithelial phenotype and/or in the onset and progression of breast cancer.

345 In summary, this study shows that RUNX1 occupies RNA Pol I- and II-transcribed
346 genes during mitosis for coordinate regulation of normal mammary epithelial proliferation,
347 growth, and phenotype. Disruption of RUNX1 DNA binding leads to the epithelial-to-
348 mesenchymal transition, a key event in breast cancer onset, and implicates RUNX1
349 mitotic gene bookmarking as an epigenetic mechanism to physiologically sustain the
350 mammary epithelial phenotype.

351 **MATERIALS AND METHODS**

352 **Cell Culture Techniques**

353 Breast epithelial (MCF10A) cells were cultured in DMEM/F-12 50/50 mixture
354 (Corning™, Corning, NY). Culturing media was also supplemented with horse serum to
355 5% (GIBCO, Grand Island, NY), human insulin to 10µg/mL (Sigma Aldrich, St. Louis, MO),
356 human epidermal growth factor to 20ng/mL (PeproTech, Rocky Hill, NJ), cholera toxin to
357 100ng/mL (Thomas Scientific, Swedesboro, NJ), hydrocortisone to 500ng/mL (Sigma
358 Aldrich, St. Louis, MO), Penicillin-Streptomycin to 100U/mL (Thermo Fisher Scientific,
359 Ashville, NC), and L-Glutamine to 2mM (Thermo Fisher Scientific, Ashville, NC).

360 For mitotic arrest of parental MCF10A cells, culturing media was supplemented
361 with 50ng/mL of Nocodazole (Sigma Aldrich, St. Louis, MO) and incubated with cells for
362 16hrs. Supplementing culturing media with equivalent volumes of DMSO (Sigma Aldrich,
363 St. Louis, MO) served as a control. DMSO-treated and mitotically arrested populations of
364 MCF10A cells were harvested following the 16hr incubation. For G1 (released from
365 mitotic arrest) populations of MCF10A cells, the nocodazole-supplemented media was
366 replaced with normal media and cells were incubated for 3hrs, after which the released
367 cell population was harvested for subsequent analyses that include western blotting,
368 qPCR, FACS and ChIP-seq.

369

370 **Western Blot Analyses**

371 Protein lysates were prepared by incubating cells in RIPA buffer on ice for 30min,
372 followed by sonication using Q700 Sonicator (QSonica, Newtown, CT). Proteins were
373 resolved by SDS-PAGE and transferred to PVDF membrane using standard protocols.

374 Following primary antibodies were used at a 1:1000 dilution (except Lamin B, which was
375 used at 1:2000 dilution) in this study: UBF (sc-13125, Santa Cruz Biotechnology, Dallas,
376 TX); RUNX1 (4334S, Cell Signaling Technologies, Danvers, MA); Cyclin B (4138S, Cell
377 Signaling Technologies, Danvers, MA); Beta-Actin (3700S, Cell Signaling Technologies,
378 Danvers, MA), and CDT1 (ab70829, AbCam, Cambridge, UK); Lamin B1 (ab16048,
379 AbCam, Cambridge, UK). Horseradish peroxidase conjugated secondary antibodies used
380 in this studies were: goat anti-mouse IgG at 1:5000 dilution (31460, Invitrogen, Carlsbad,
381 CA), goat anti-rabbit IgG HRP conjugated (31430, Thermo Fisher Scientific, Ashville, NC)
382 at 1:1000, 1:2000, or 1:5000 dilutions. Blots were developed using Clarity Western ECL
383 Substrate (Bio-Rad, Hercules, CA) and imaged using Molecular Imager® Chemi doc™
384 XRS+ Imaging System (Bio-Rad, Hercules, CA) aided by Image Lab Software Version
385 5.1 (Bio-Rad, Hercules, CA).
386
387 **Confocal Microscopy, Image Acquisition, Processing and Analyses**
388 MCF10A cells were plated on gelatin-coated coverslips in 6-well plates at 175,000
389 cells/mL and processed for immunofluorescence 24 hrs after plating using standard
390 protocol. Briefly, cells were washed twice with sterile-filtered PBS on ice and cell were
391 fixed in 1% MeOH-free Formaldehyde in PBS for 10min. After permeabilization in 0.25%
392 Triton X-100-PBS solution, cells were sequentially incubated with primary and Alexa
393 fluorophore conjugated secondary antibodies for 1hr each at 37℃ in a humidified
394 chamber with extensive washes after each incubation. Primary antibodies used were:
395 RUNX1 at 1:10 dilution (4334S, Cell Signaling Technologies, Danvers, MA), and
396 Upstream Binding Transcription Factor (UBF) at 1:200 dilution (F-9 sc-13125, Santa Cruz

397 Biotechnology, Dallas, TX). Secondary antibodies used were goat anti-rabbit IgG
398 conjugated with Alexa Fluor 488 (A-11070, Life Technologies, Carlsbad, CA) and goat
399 anti-mouse IgG conjugated with Alexa Fluor 594 (A-11005, Life Technologies, Carlsbad,
400 CA) diluted 1:800. Cells were counterstained with DAPI to visualize DNA and coverslips
401 were mounted onto slides in ProLong Gold Antifade Mountant (Thermo Fisher Scientific,
402 Ashville, NC). Images were captured using a Zeiss Axio Imager.Z2 fluorescent
403 microscope and Hamamatsu ORCA-R2 C10600 digital camera. Images were processed
404 using ZEN 2012 software.

405 To examine mitotic localization of RUNX1 in unfixed cells, an expression plasmid
406 containing RUNX1-EGFP was introduced using either nucleofection or Lipofectamine
407 2000 transfection reagent in actively proliferating MCF10A cells grown on gelatin-coated
408 coverslips. After 16 hours of nucleofection, cells were washed once with 1X PBS and
409 stained with Hoechst dye to visualized DNA. Coverslips were mounted using the ProLong
410 Gold Antifade Mountant and subjected to confocal microscopy.

411 Cells were initially imaged with a Zeiss LSM 510 META confocal laser scanning
412 microscope (Carl Zeiss Microscopy, LLC., Thornwood, NY, USA). The DAPI signal was
413 excited with a 405 nm laser and collected with a 425-475 nm band pass filter, Alexa 488
414 was excited with a 488 nm laser and collected with a 500-550 nm band pass filter, and
415 Alexa 568 with a 561 nm laser and collected with a 570-620 nm band pass filter. Images
416 were captured with a Plan-Fluor 40X (1.3 NA) objective lens. The confocal pinhole was
417 initially set to 1.2 Airy Unit diameter for the 561 nm excitation giving an optical section
418 thickness of 0.41 μm. Images were acquired at 12-bit data depth, and all settings,
419 including laser power, amplifier gain, and amplifier offset were established using a look

420 up table to provide an optimal gray-scale intensities. All images were acquired using
421 matching imaging parameters. The acquired images were subjected to colocalization
422 analysis via Volocity version 6.3.0 (Perkin Elmer, Waltham, MA, USA). At least 15
423 interphase and 15 metaphase cells were identified in captured images and appropriate
424 thresholds were manually determined to eliminate background fluorescence for
425 calculating Pearsons and Manders correlation coefficients between RUNX1 and UBF.

426 To ensure the specificity of our observations, additional samples were imaged with
427 a Nikon A1R-ER laser scanning confocal microscope (Nikon, Melville, NY, USA). Images
428 were acquired with the galvano scanner at a frame size of 1024 X 1024 pixels with an
429 Apo TIRF 60X objective lens (N.A. 1.49) zoom of 2 and 1.2 Airy Unit pinhole setting.
430 Images were also viewed in NIS Elements version 5.02.01 and analyzed using the line
431 profiling tool. Overlaying DAPI, RUNX1, and UBF fluorescent intensities from individual
432 channels along the line profile revealed overlapping peak intensities between the RUNX1
433 and UBF channels, thus indicating colocalization.

434

435 **RNA Isolation, cDNA Synthesis and Quantitative PCR**

436 Total RNA was isolated from MCF10A cells using TRIzol™ Reagent (Invitrogen,
437 Carlsbad, CA) and Direct-Zol™ RNA MiniPrep isolation kit (Zymo Research, Irvine, CA)
438 following manufacturer instructions. cDNA was created using SuperScript IV® First-
439 Strand Synthesis System for RT-PCR (ThermoFisher, Asheville, NC). Resulting samples
440 were quantified on a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and diluted to
441 500pg/μL. Equal amounts of DNA template were loaded for samples analyzed by qPCR.
442 At least three independent biological replicates were analyzed for expression of RUNX1

443 bookmarked genes by qPCR. Student's t-test was used to determine the significance of
444 changes in transcript levels under different biological conditions.
445

446 **Chromatin Immunoprecipitation, Library Preparation, Sequencing and**
447 **Bioinformatics Analyses**

448 Asynchronous (Asynch), mitotically arrested (M), and released from mitosis (G1)
449 MCF10A breast epithelial cells were subjected to chromatin immunoprecipitation using a
450 modified Farnahm' protocol {O'Geen, 2010 #2713}. Sonication parameters for each
451 population of cells was as follows: Peak Watt 140W, Duty Factor 10, Cycle/Burst 200
452 using a S220 focused ultra-sonicator (Covaris, Matthews, NC). M and G1 populations of
453 cells were sonicated for 28min total, whereas asynchronous populations of cells were
454 sonicated for 36min. An aliquot of sonicated lysates was boiled in 100°C for 15min with
455 NaCl and elution buffer and DNA was purified using PureLink^{TM} PCR Purification Kit
456 (K310001, ThermoFisher, Ashville, NC). Purified DNA was resolved on a 1.5% agarose
457 gel to confirm optimal sonication (bulk of fragments between 200-400bp) prior to
458 performing ChIP. In parallel, an aliquot was also quantified via Qubit 2.0 Fluorometer
459 (Invitrogen, Carlsbad, CA) and analyzed by using a High Sensitivity DNA Kit on a
460 Bioanalyzer 2100 (Agilent, Santa Clara, CA).

461 For chromatin immunoprecipitation (ChIP) reactions, 150ug of sonicated
462 chromatin was incubated with 10ug of RUNX1 antibody (4336BF, Cell Signaling
463 Technologies, Danvers, MA), diluted 1:10 in IP dilution buffer, and incubated overnight
464 (16-18hrs) at 4°C with mild agitation. Following incubation, 150uL of Protein A/G magnetic
465 beads (Thermo Scientific – Pierce, Waltham, MA) per ug of antibody used were added to

466 each IP reaction and incubated for 2-4hrs at 4ºC with mild agitation. Beads were
467 extensively washed with IP wash buffers and resuspended in Elution Buffer to extract
468 immunoprecipitated chromatin, which was subsequently purified using PureLink™ PCR
469 Purification Kit. At least 3 biological replicates were performed for each cell population
470 and each antibody.

471 ChIP libraries were generated using Accel-NGS® 2S Plus DNA Library kit (Swift
472 Biosciences, Ann Arbor, MI) following manufacturers protocol. Input and RUNX1 ChIP
473 samples were normalized to 1ng prior to library generation. Libraries were amplified in an
474 optional PCR step for 12 total cycles. Finalized libraries were double size selected using
475 AMPure XP beads (0.8X and 0.2X volume ratios to sample), resulting in the majority
476 fragments sized between 250-400bp. Next generation sequencing of pooled ChIP
477 libraries was performed by the University of Vermont Cancer Center - Vermont Integrated
478 Genomics Resource (VIGR).

479 Single end, 50bp reads (SE50) were processed pre-alignment by removing
480 adapter reads (Cutadapt v1.6) and trimming low quality base calls from both ends
481 (FASTQ Quality Trimmer 1.0.0; min score >= 20, window of 10, and step size of 1).
482 Because we were specifically investigating rDNA, a customized build of hg38 was
483 constructed that included normally masked regions of rDNA (Gencode U13369). Since
484 some (although not complete) rDNA sequence is present in the hg38 assembly, we
485 masked all parts of hg38 that would normally be attributed to rDNA sequences (bedtools
486 v2.25.0 maskfasta) based on alignment positions of 50 bp *in silico* reads generated
487 across U13369. Finally, we appended the complete rDNA sequence as a distinct

488 sequence (chrU13369.1) to the masked hg38 FASTA resulting in the hg38_rDNA
489 assembly used for analysis.

490 Resulting reads were aligned to hg38_rDNA (STAR v2.4; splicing disabled with '--
491 alignIntronMax 1'). Next, we called peaks and generated fold-enrichment (FE) bedGraph
492 files (MACS2 v2.1.0.20140616; callpeak at p-value e-5; and bdgcmp with FE
493 method)(98). Irreproducible Discovery Rate (IDR) was conducted using unpooled
494 replicates with all peaks in pooled samples passing an IDR cutoff of 0.5 (99). To reduce
495 artificial peaks, we calculated strand cross-correlation for all peaks at a shift of 95 bp (the
496 mean observed fragment size of 180 bp minus the read size of 85bp) and unshifted (100).
497 We eliminated peaks that exhibited low shifted correlation (shifted correlation <0.7) and
498 those that exhibited high unshifted correlation relative to shifted (shifted – unshifted
499 correlation < 0.1). This increased retrieval of the RUNX1 motif and improved agreement
500 with other RUNX1 datasets. Passing peaks were annotated separately to mRNA and
501 lncRNA transcript start sites (TSSs) using Gencode v27 with a distance cutoff of 5000 bp.
502 Regional distribution of peaks was determined using the same annotation reference
503 limited to the "basic" tag for exons and promoters.

504

505 **Inhibitor Treatment and Assessment of Global Protein Synthesis**

506 Core binding factor – Beta (CBFβ) inhibitors AI-4-88 and AI-14-91 were kindly
507 provided by John H. Bushweller (University of Virginia) and used to evaluate RUNX1
508 DNA-binding inhibition in MCF10A cells. Protein synthesis evaluation by
509 immunofluorescence was conducted following manufacturer protocol (K715-100,
510 BioVision, San Francisco, CA). To examine effects of inhibiting the RUNX1-CBFβ

511 interaction, MCF10A cells were treated with active or inactive compound for 48 hours.
512 Culture medium containing the active or inactive compounds was replaced with fresh
513 medium without the compounds and cells were harvested 4- and 7-days post medium
514 change.
515
516 **RNA-Sequencing, Differential Expression Analysis, and Pathway Analysis:**
517 RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA,
518 USA) and was quantified and assayed for RNA integrity by Bioanalyzer (Agilent
519 Technologies, Inc., Santa Clara, CA, USA). Following the removal of ribosomal RNA, the
520 RNA pool was reverse transcribed, amplified, purified, and bound to strand-specific
521 adaptors following the manufacturer's protocol (SMARTer Stranded Total RNA Sample
522 Prep Kit, Takara Bio, Mountain View, CA, USA). cDNA libraries were assayed for quality
523 control by Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). After cDNA
524 quality validation, generated libraries were sequenced. 24 hour and 48-hour counts were
525 grouped together into one "crisis" category and the day 4 recovery and day 7 recovery
526 counts were grouped together into one "recovery" category. Treatment groups were
527 compared with untreated MCF10A cells. After demultiplexing and quality filtering, reads
528 were aligned to hg38 using Gencode (GRCh38.p13). As a reference, annotation with
529 STAR (v2.5.2a)(101) aligned reads were then counted using HT-Seq (102). Differential
530 gene expression was analyzed using DESeq2 in R v.3.5.1 (103). Parameters for
531 significant differential expression were base mean expression greater than five, absolute
532 log2 fold change greater than one, and a p-value less than 0.05. Pathway analysis was

533 performed using Gene Set Enrichment Analysis v6.3 (Broad Institute, Inc., MIT, UC San
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535

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544

545 **COMPETING INTERESTS**

546 No competing interests declared.
547

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559

560 **DATA AVAILABILITY**

561 GEO accession number for the sequencing data generated in this study is
562 GSE121370.

563 **FIGURE LEGENDS**

564 **Figure 1. RUNX1 associates with DNA during interphase and remains bound**
565 **throughout mitosis in the form of major and minor foci.** Representative
566 immunofluorescent images of interphase and mitotic MCF10A breast epithelial cells
567 showing subcellular localization of RUNX1, identified using a specific antibody,
568 throughout mitosis. Mitotic cells were further classified into substages of mitosis based
569 on DAPI topology. RUNX1 – Green (top row), DAPI – Blue (second row from top).
570 Merged channel images (third row from top) contain an outlined region magnified in the
571 bottom row labeled "inset". White arrows highlight major Runx1 foci on mitotic
572 chromatin. Three independent biological replicates were performed, and at least 20
573 cells for each mitotic substage were analyzed.

574

575 **Figure 2. RUNX1 occupies protein coding genes and long non-coding RNAs**
576 **across asynchronous, mitotic, and G1 populations of MCF10A breast epithelial**
577 **cells.** A) Heatmaps showing peaks called between A, M, and G1 MCF10A cells (left,
578 middle, and right respectively). Cumulative occupancy of RUNX1 is shown as line
579 graphs and genes that occupied by RUNX1 in each of the three cell populations are
580 shown. Shown also are genes that are deregulated upon RUNX1 downregulation. For
581 example, 1070 genes are bound by RUNX1 in cells that are neither in M nor G1 cells
582 (green bar graph) and of those, 353 genes are deregulation upon RUNX1 deregulation.
583 B) Gene ontology analysis of RUNX1-bound genes identifies key regulatory pathways
584 that are similar or unique in the three cell populations. C) Motif analysis of A, M, and G1
585 MCF10A cells reveals RUNX motif as one of the top motifs in all cell populations.

586 Binding sites for key transcription factors that are known to cooperate with RUNX1 are
587 also identified.

588 **Figure 3. RUNX1 bookmarks rDNA promoter repeat regions and affects both pre-**
589 **rRNA and global protein expression.** A) ChIP-Seq tracks of A, M, and G1 (top,
590 middle, bottom respectively) MCF10A cells mapped against rDNA repeat regions. B) A
591 representative metaphase MCF10A cell, stained for RUNX1 (green) and UBF1 (red)
592 localization, is shown demonstrating that the two proteins colocalize during mitosis
593 (merged). Cells are also counter stained with DAPI to visualize DNA (blue) and identify
594 mitosis substages. C) qRT-PCR data of pre-rRNA in actively proliferating MCF10A cells
595 treated with either active (AI-14-91) or inactive (AI-4-88) compounds for 6, 12, 24, or
596 48hrs. Expression of pre-rRNA was normalized relative to Beta Actin expression. Graph
597 represents three independent biological replicates. Asterisks represents a *p* value of
598 <0.05. D) Representative fluorescence microscopy images of global protein synthesis
599 from MCF10A cells treated with either AI-4-88 (left) or AI-14-91 (right) for 24hr at 20μM
600 (n=2). Intensity of red fluorescence at 580nm emission indicates nascent protein
601 synthesis. All images were taken with 1000ms exposures.

602 **Figure 4. RUNX1 bookmarks RNA Pol II-transcribed genes involved in**
603 **maintenance of breast epithelial phenotype.** A) Gene Set Enrichment (GSE) analysis
604 from interrogating mitotically bookmarked genes (i.e. RUNX1 mitotically occupied)
605 against Hallmark Gene sets from Molecular Signatures Database (MSigDB). The top 10
606 most significantly overlapping gene sets are shown from top to bottom. B) Scatter plot of
607 genes identified to be up or down regulated in response to estradiol treatment, that are
608 also bound by estrogen receptor α (ERα) and RUNX1 (empty circles, blue for

609 downregulated and red for upregulated). Scatter plot also illustrates up or down
610 regulated genes in response to estradiol treatment that are bound by ERα and
611 mitotically bookmarked by RUNX1 (filled in circles, blue for downregulated and red for
612 upregulated). C) Top panel: ChIP-Seq tracks of *HES1* (left) and *H2AFX* (right) from
613 asynchronous (top-red), mitotic (middle-green), and G1 (bottom-blue). Bottom panel:
614 qRT-PCR data of *HES1* (left) and *H2AFX* (right) in asynchronous MCF10A cells treated
615 with either active (AI-14-91) or inactive (AI-4-88) inhibitors for 6hr, 12hr, 24hr and 48hr
616 at 20μM. Expression of target genes were normalized relative to beta actin.

617 **Figure 5. Disrupting RUNX1 mitotic gene bookmarking in MCF10A cells leads to a**
618 **transformed cellular phenotype and EMT.**

619 A) Phase contrast microscopy images of MCF10A cells treated with AI-4-88 or A-14-91
620 for 48 hours at 20μM. Left panel – 20X magnification, right panel – 40X magnification.
621 The outlines rectangle in the left panel is the resulting 40X magnification in the right
622 panel. B) Top panel: Experimental schematic depicting treatment schedule for the
623 "crisis" and "recovery" stages. Bottom panel: Phase contrast microscopy images from
624 Day 0, 1, and 2 or crisis where MCF10A cells were treated with AI-14-91 at 20μM (top –
625 left, middle, right respectively). Phase contrast images from Day 0, 4, and 7 or recovery
626 following a media replacement. C) Morphological changes upon inhibition of the
627 cytoskeletal protein F-actin. When compared to inactive compound (left panel), cells
628 treated with the active compound show substantial alteration in cytoarchitecture (right
629 panel). D) Western blot for RUNX1, RUNX2, epithelial marker E-cadherin,
630 mesenchymal marker Vimentin, and loading control Beta-actin (top panel to bottom

631 panel respectively) in MCF10A whole cell lysate harvested from the crisis 24 hour and
632 48-hour timepoints and recovery day 4 and day 7 timepoints.

633 **Figure 6. Differential expression and pathway analysis of RNA-Seq shows**
634 **changes in key regulatory pathways involved in cell proliferation, metabolism,**
635 **cell cycle control, estrogen response, and EMT.** A) Expression heatmap of three
636 biological replicates of 24-hour and 48-hour crisis timepoints and Day 4 and Day 7
637 recovery timepoints. B) Scatterplot of log2 fold change between crisis and recovery
638 phases. Most changed genes in each stage are indicated. C) Table of overlapping
639 pathways specific to crisis and recovery stages. D) Representative immunofluorescence
640 images of the active compound (AI-14-91)-treated MCF10A cells in prophase and
641 metaphase are shown. A substantial decrease in smaller RUNX1 foci (green) during
642 mitosis is observed when compared to the inactive (AI-4-88) compound. Large RUNX1
643 foci that colocalize with UBF (red) are detectable at all substages of mitosis (white
644 arrows) in the presence of either active or inactive compounds.

645 **Supplementary Figure 1. Secondary antibody controls for immunofluorescence**
646 **microscopy.** To ensure the specificity of RUNX1 signal on mitotic chromosomes,
647 actively proliferating mammary epithelial MCF10A cells, grown on gelatin-coated
648 coverslips, were subjected to immunofluorescence microscopy procedure as described
649 in Materials and Methods section with one modification: no primary antibody was added,
650 but secondary antibodies were used at the same dilution as in all IF experiments. Nuclei
651 were counterstained with DAPI. Two different fields are shown, confirming that the
652 RUNX1 signal we observe on mitotic chromosomes is specific.

653 **Supplementary Figure 2. RUNX1 associates with chromosomes during all stages**
654 **of mitosis in unfixed live MCF10A cells.** Mammary epithelial MCF10A cells were
655 transiently transfected with EGFP-RUNX1 and imaged by confocal microscopy without
656 fixation (see Materials and Methods section for details). Top panels show RUNX1
657 (green) association with mitotic chromosomes in unfixed, live MCF10A cells. Cells were
658 counterstained with Hoechst (middle panels; blue) to visualize DNA in live cells and to
659 identify mitotic cells. Merged images (bottom panels) were generated to confirm
660 localization of RUNX1 signal with DNA. Arrow heads indicate punctate RUNX1 foci
661 retained on mitotic chromosomes.

662 **Supplementary Figure 3. Determination of optimal nocodazole dosage for**
663 **maximum mitotic block.** A) Micrographs of MCF10A cells, treated with various doses
664 of nocdazole for 16 hours, are shown (bottom panels). DMSO control treatment is also
665 included (top panels). B) Experimental schematic depicting mitotic arrest and harvest of
666 each treated MCF10A cell population: Asynchronous – A, Mitotic – M, and Released –
667 G1. C) Western blot of each harvested MCF10A population for cell cycle specific
668 markers to evaluate mitotic arrest and synchronization procedure. D) Fluorescence-
669 activated cell sorting (FACS) analysis of harvested A, M, and G1 MCF10A populations
670 to determine mitotic purity and DNA content (n=2 biological replicates per group).

671 **Supplementary Figure 4. RUNX1 colocalizes with RNA Pol I subunit, upstream**
672 **binding factor (UBF) on mitotic chromatin.** A) Immunofluorescence microscopy
673 images of RUNX1 (green – top row), UBF (red – 2nd row from top), DAPI (blue – 2nd row
674 from bottom), and the three channels merged (bottom row) in MCF10A cells. Images
675 were captured of spontaneously dividing MCF10A cells in different substages of mitosis.

676 B) Representative images of line profiles taken on interphase vs metaphase cells (n=15
677 each).

678 **Supplementary Figure 5. The RUNX1-CBFβ inhibitor reduces RUNX1 association**
679 **with mitotic chromosomes.** MCF10A cells, treated with 20μM inactive control
680 compound or active inhibitor for 12 hours, were stained for localization of endogenous
681 RUNX1 (green) to mitotic chromosomes. RUNX1 retention on mitotic chromosomes,
682 particularly in smaller foci, was substantially reduced in cells treated with active inhibitor
683 of RUNX1-CBFβ interaction, which disrupts RUNX1 DNA binding activity.

684 **Dataset S1 (separate file).** List of genes occupied by RUNX1 in asynchronous, mitotic
685 and G1 cell populations in mammary epithelial cells.

686 **Dataset S2 (separate file).** Gene enrichment analysis of genes sensitive to RUNX1-
687 CBFβ inhibition during crisis and recovery phases of epithelial to mesenchymal
688 transition in MCF10A cells.

689

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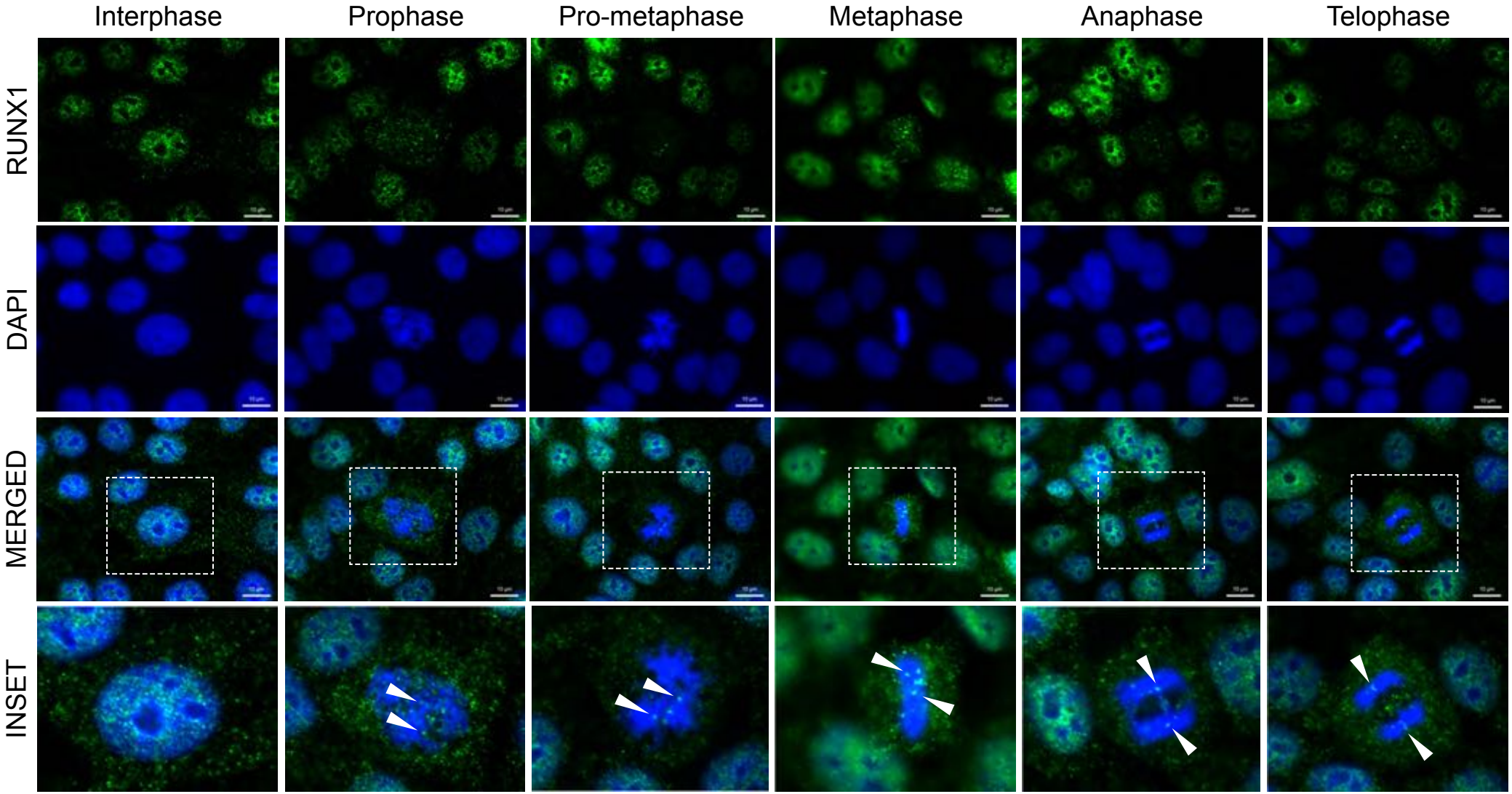

Interphase
Prophase
Pro-metaphase
Metaphase
Anaphase
Telophase
RUNX1
DAPI
MERGED
INSET

Figure 1

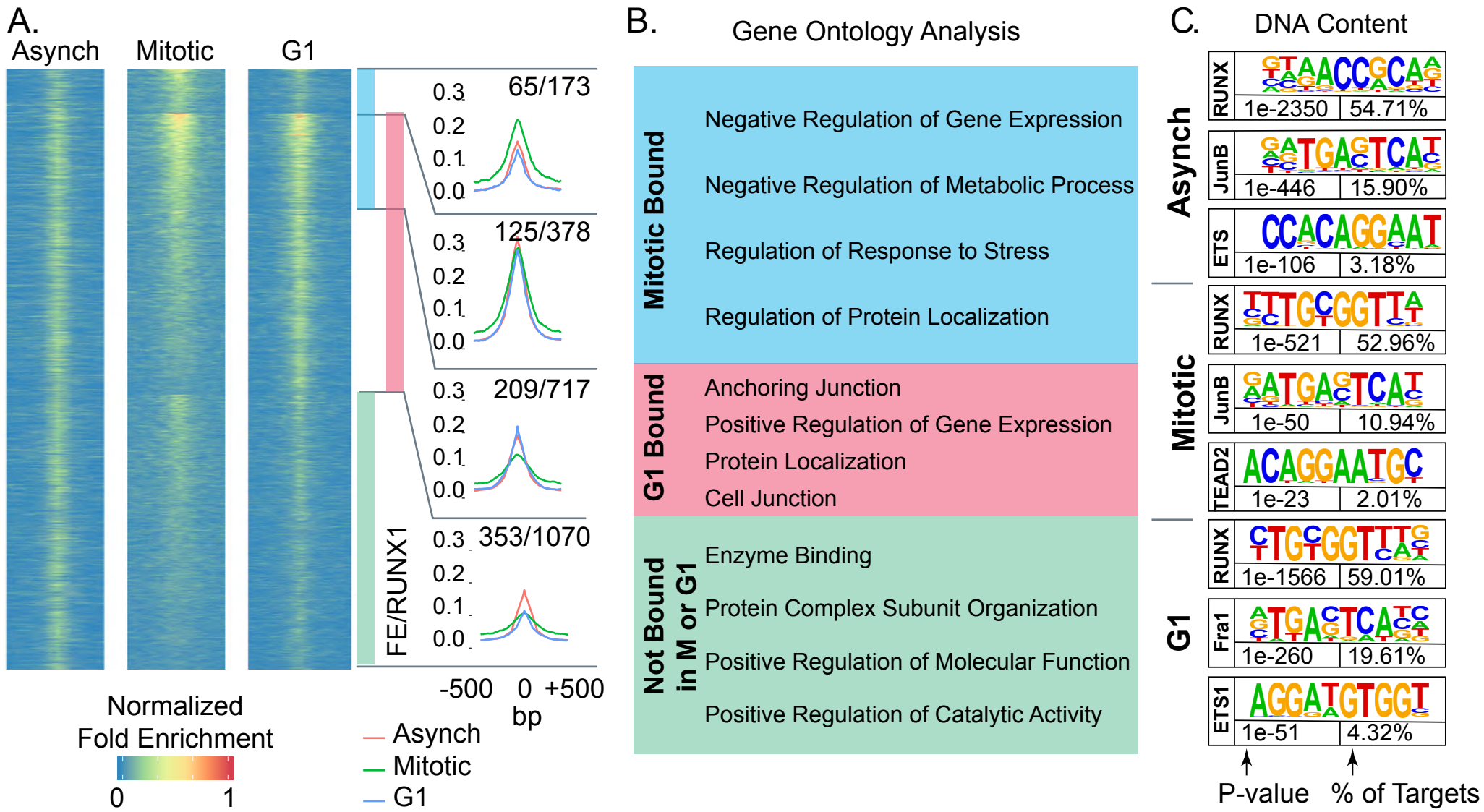
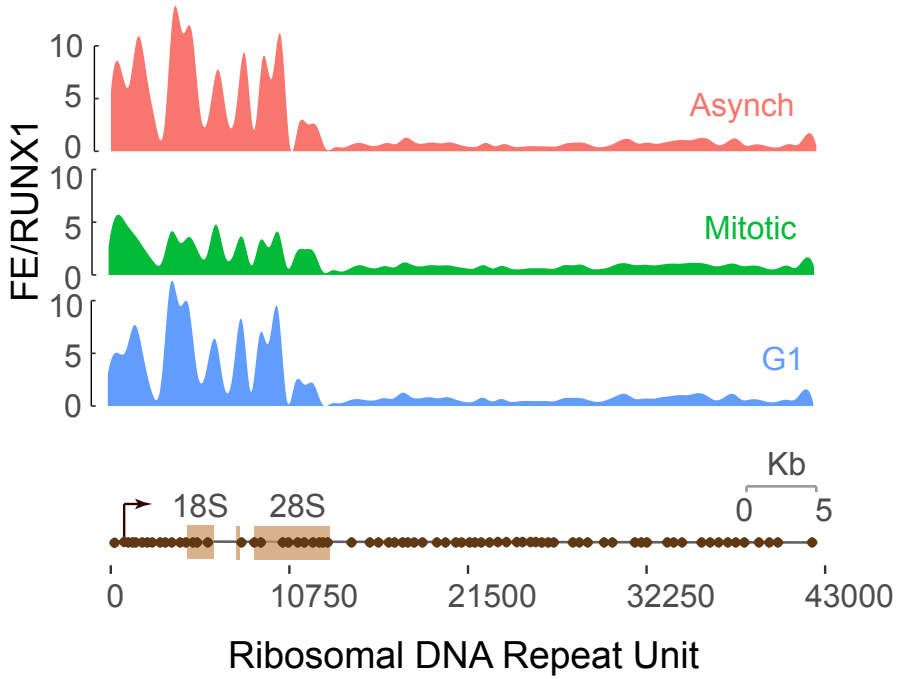
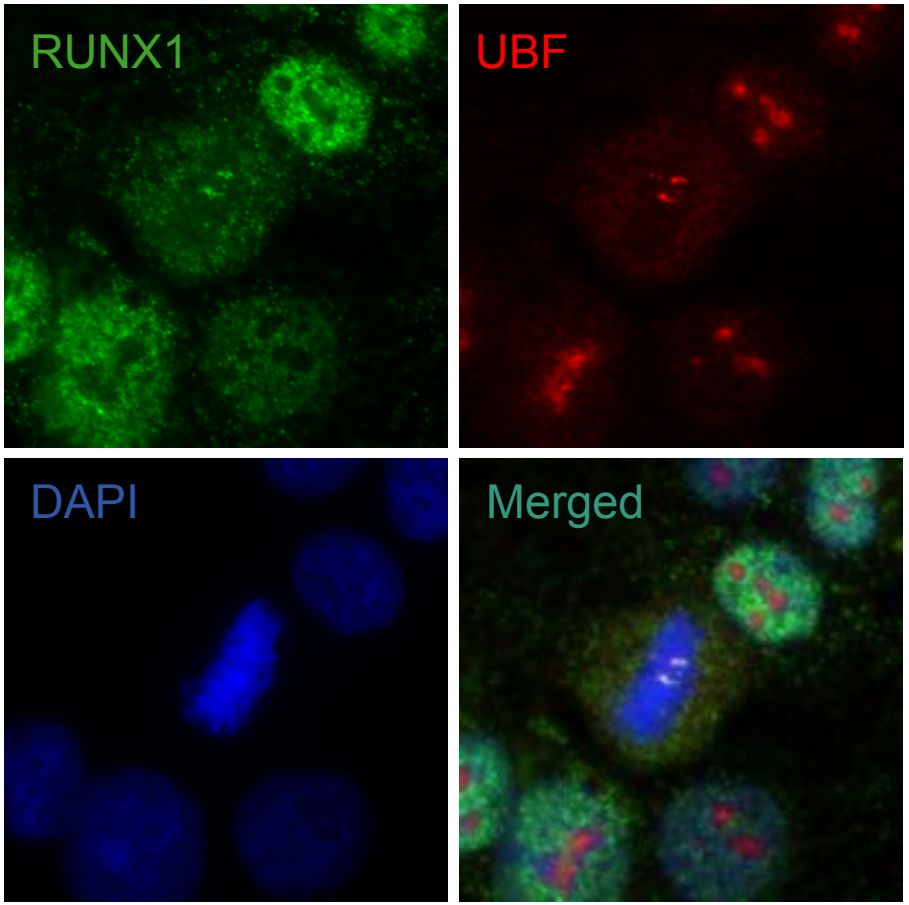

A.
Asynch
Mitotic
G1
65/173
125/378
209/717
353/1070
FE/RUNX1
0.3
0.2
0.1
0.0
-500
0
+500
bp
Normalized
Fold Enrichment
0
1
Asynch
Mitotic
G1
B.
Gene Ontology Analysis
Mitotic Bound
Negative Regulation of Gene Expression
Negative Regulation of Metabolic Process
Regulation of Response to Stress
Regulation of Protein Localization
G1 Bound
Anchoring Junction
Positive Regulation of Gene Expression
Protein Localization
Cell Junction
Not Bound in M or G1
Enzyme Binding
Protein Complex Subunit Organization
Positive Regulation of Molecular Function
Positive Regulation of Catalytic Activity
C.
DNA Content
Asynch
RUNX
1e-2350
54.71%
JunB
1e-446
15.90%
ETS
1e-106
3.18%
Mitotic
RUNX
1e-521
52.96%
JunB
1e-50
10.94%
TEAD2
1e-23
2.01%
G1
RUNX
1e-1566
59.01%
Fra1
1e-260
19.61%
ETS1
1e-51
4.32%
P-value
% of Targets

Figure 2

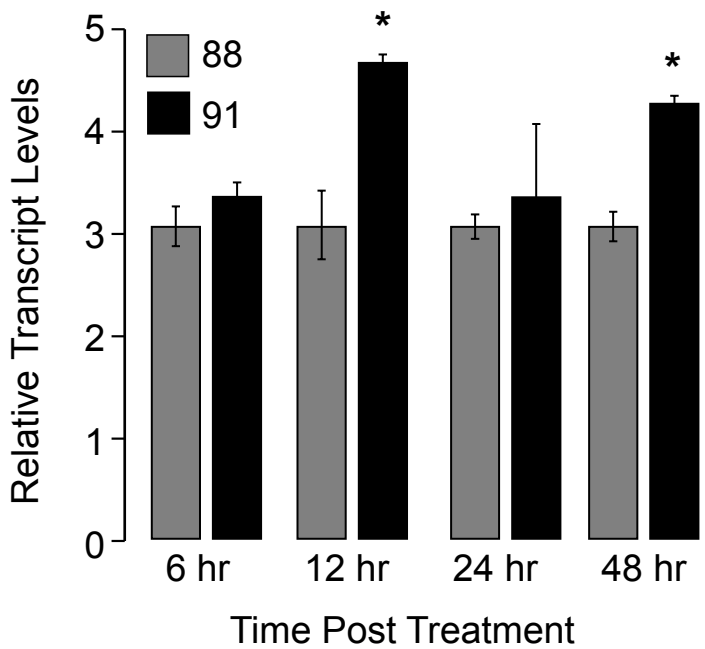
A *Genomic Tracks*



B *Immunofluorescence*



C *pre-rRNA*



D *Protein Label*

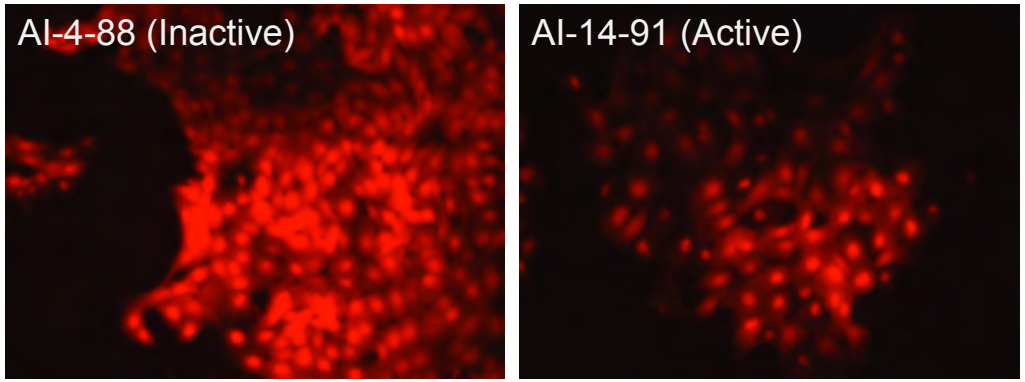
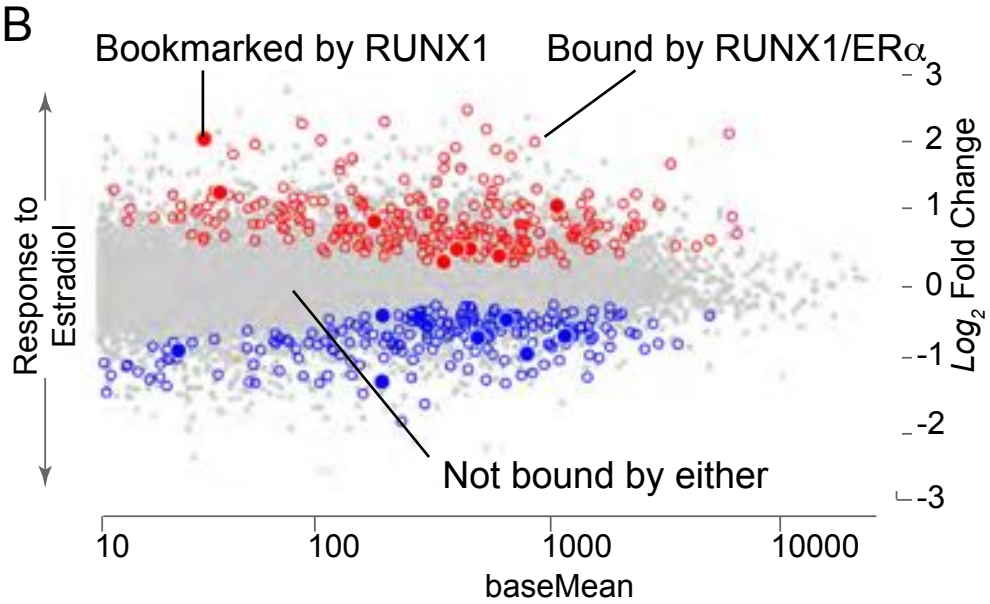


Figure 3

A

Hallmark Gene Set	# of Bookmarked Genes
Late Estrogen Response	12
mTOR Signaling	12
TNFα Signaling	12
Apical Junction	11
Early Estrogen Response	10
G2M Checkpoint	10
p53 Pathway	10
DNA Repair	8
E2F targets	9
Notch Signaling	4

B



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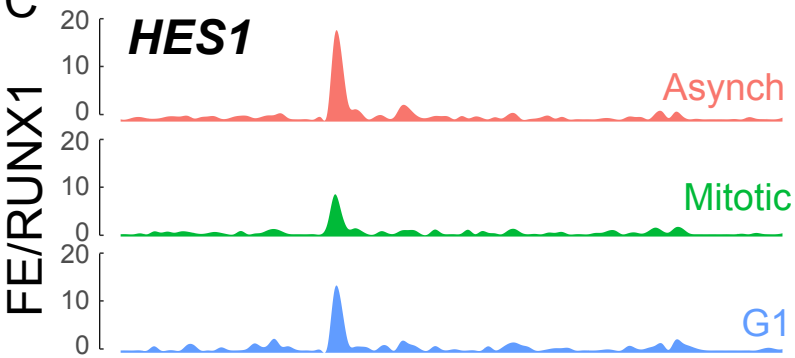
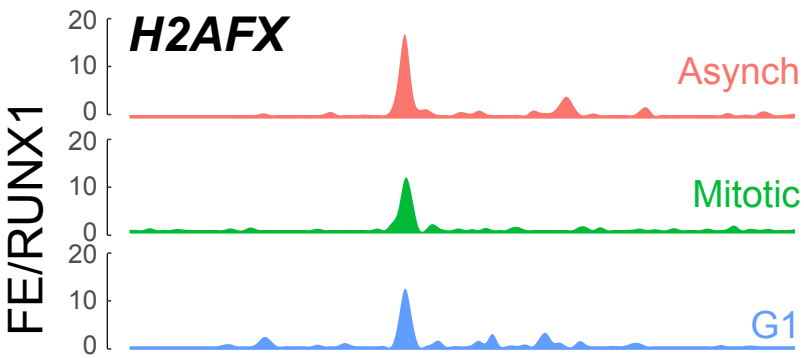
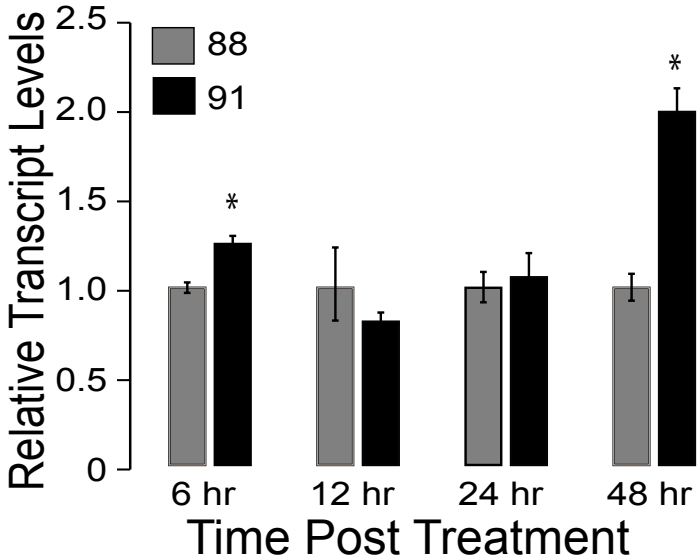






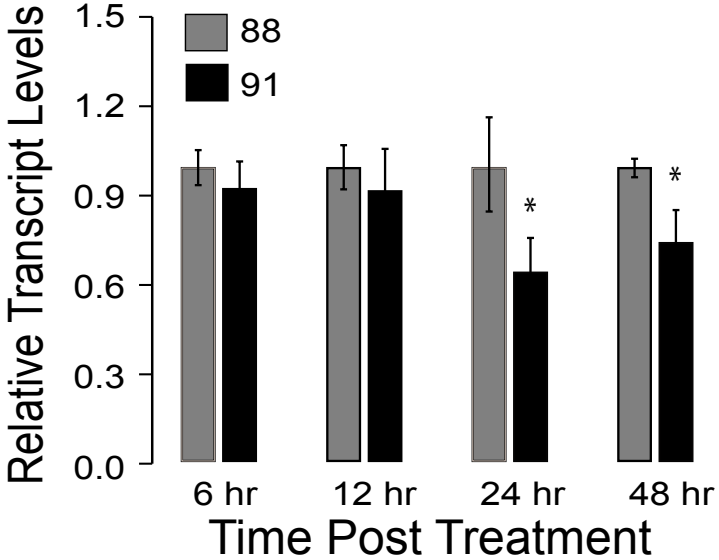
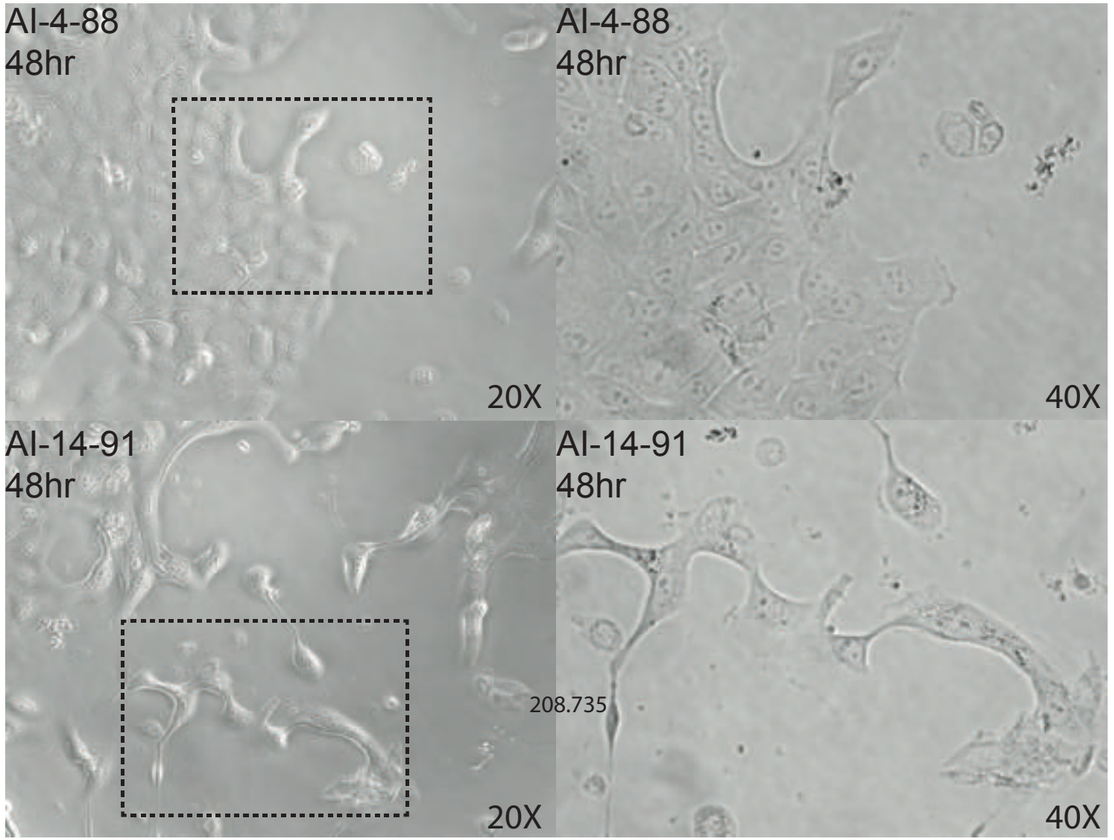
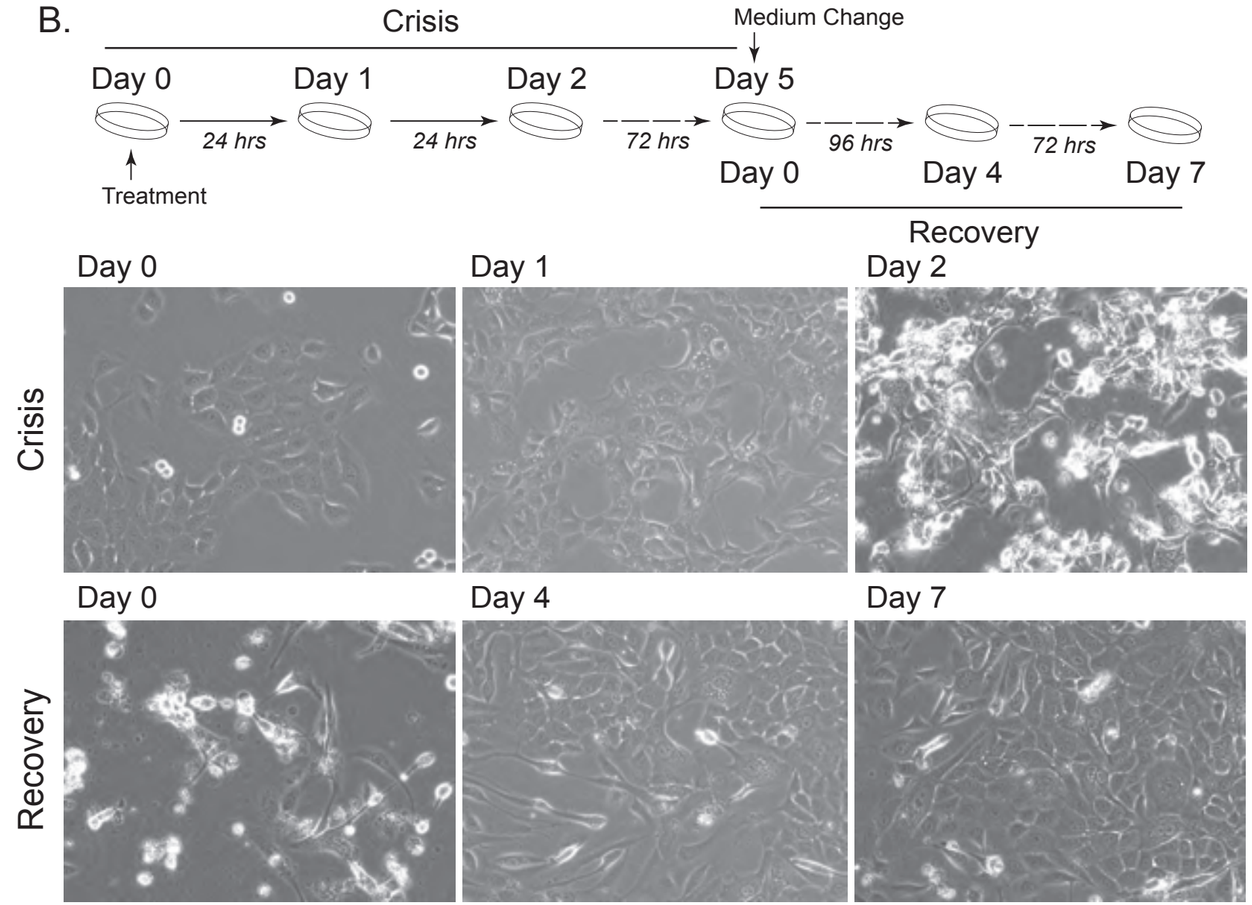


Figure 4

A.



B.



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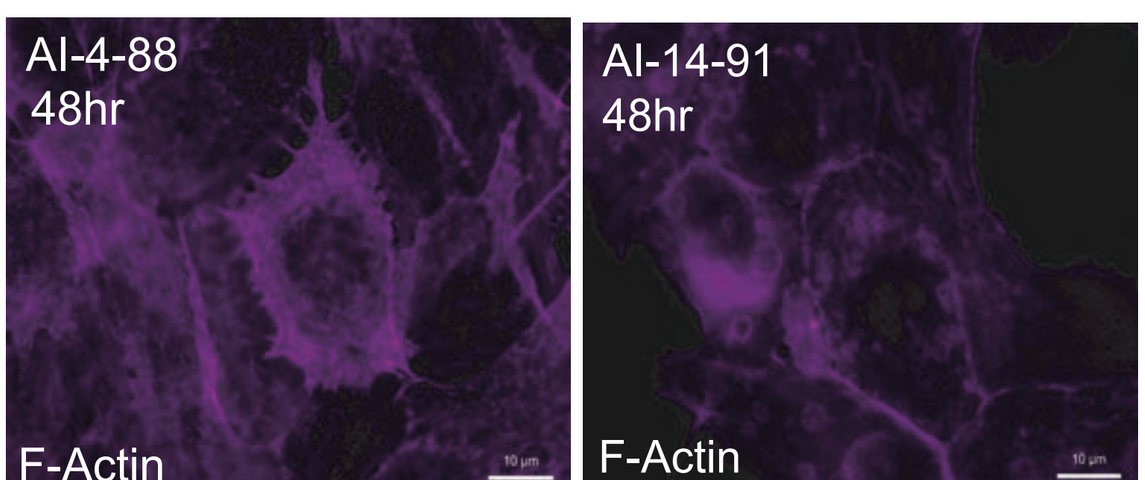


D.

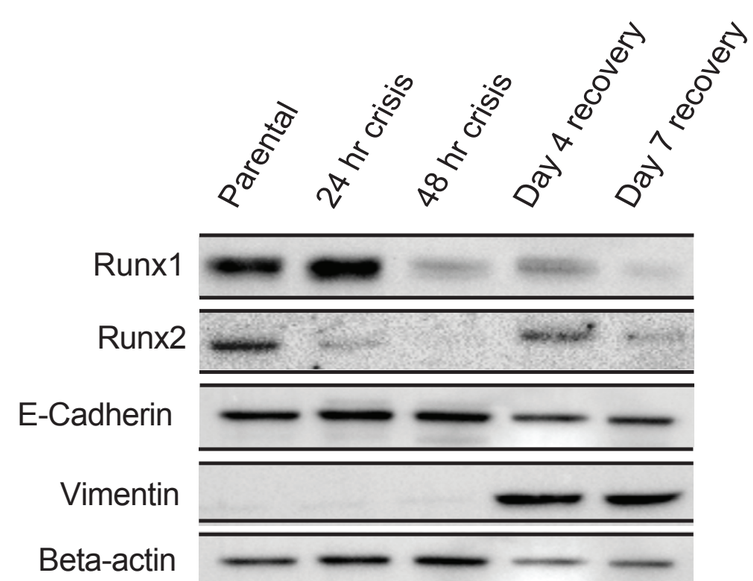
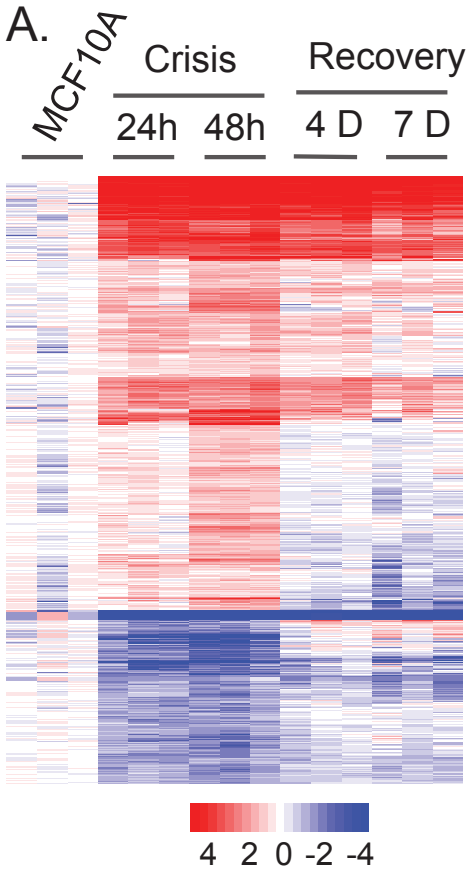


Figure 5

A.



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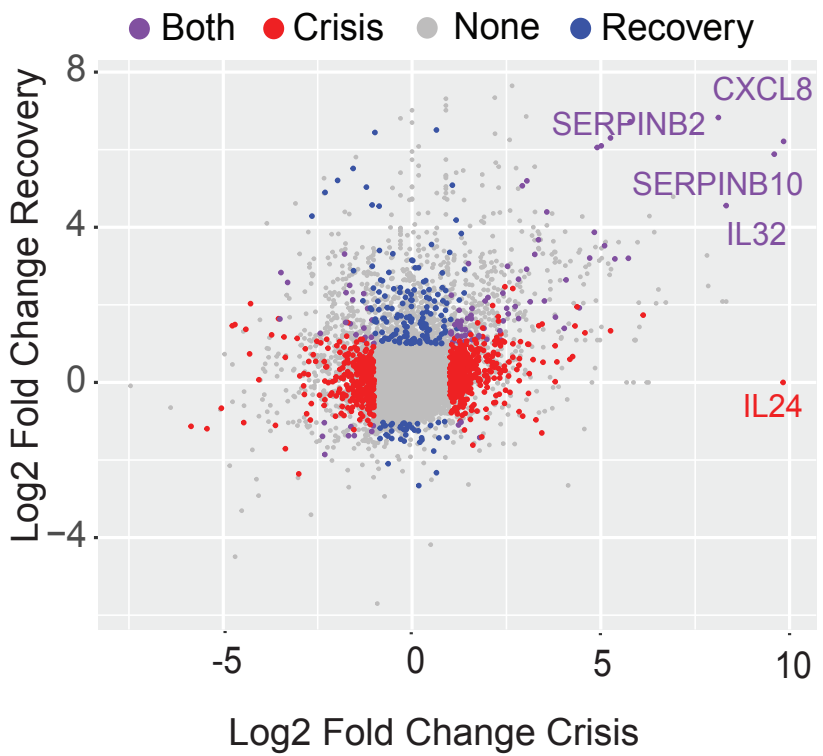


C.

Hallmark Gene Set	Overlapping Crisis DEGs	Enrichment Score
mTORC1 Signaling	35	0.50
TNFα Signaling	69	0.45
Glycolysis	32	0.40
Apoptosis	30	0.26
EMT	28	0.22
p53 Pathway	37	0.20

Hallmark Gene Set	Overlapping Recovery DEGs	Enrichment Score
IFNγ Response	21	0.41
EMT	15	0.40
Complement	19	0.36
TNFα Signaling	27	0.34
KRAS Signaling	22	0.20

D.

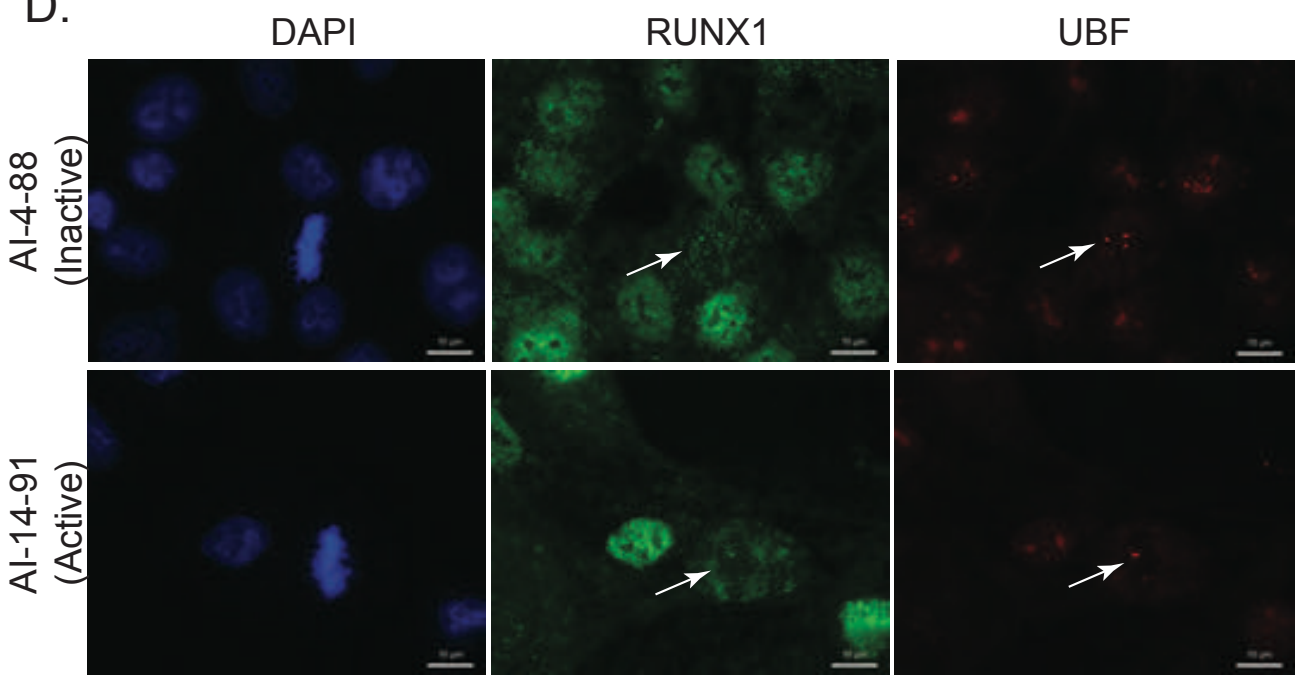
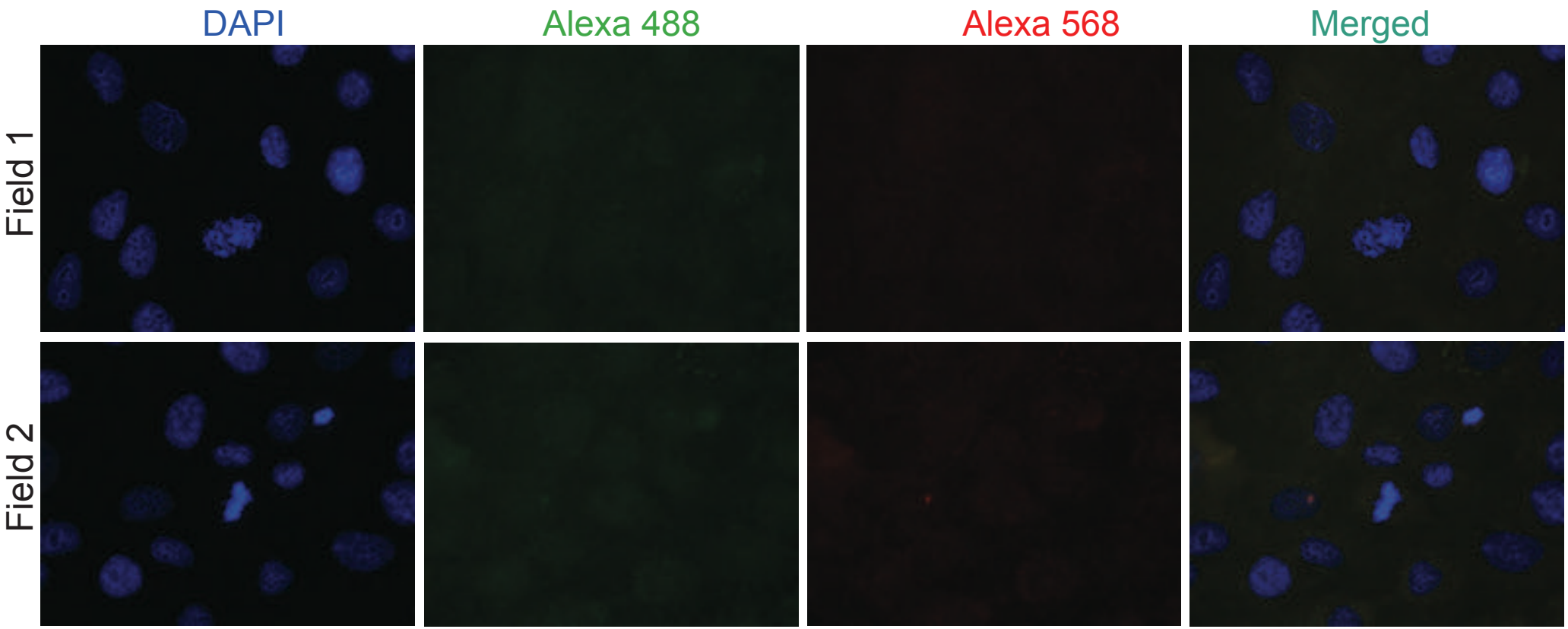
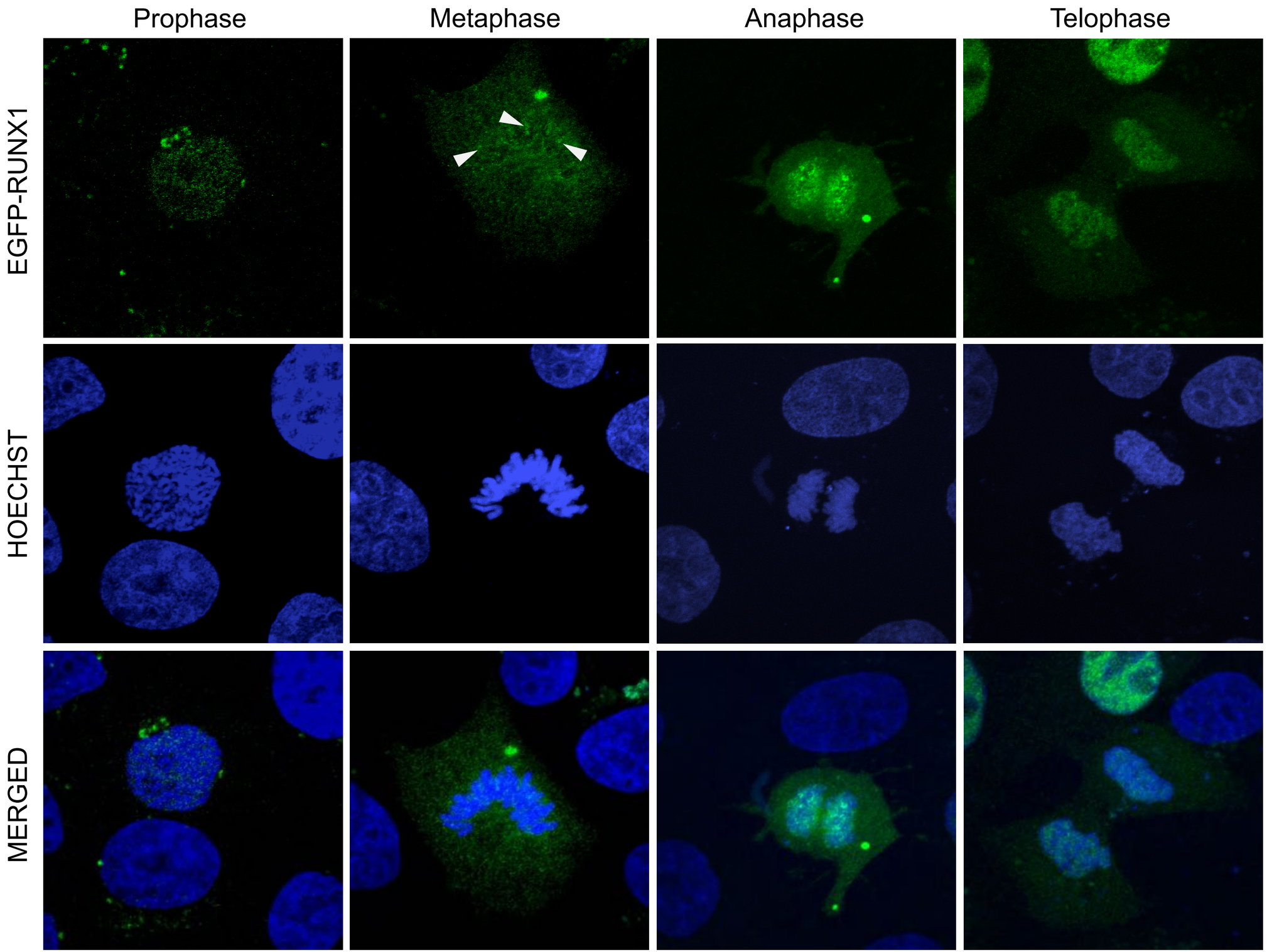


Figure 6

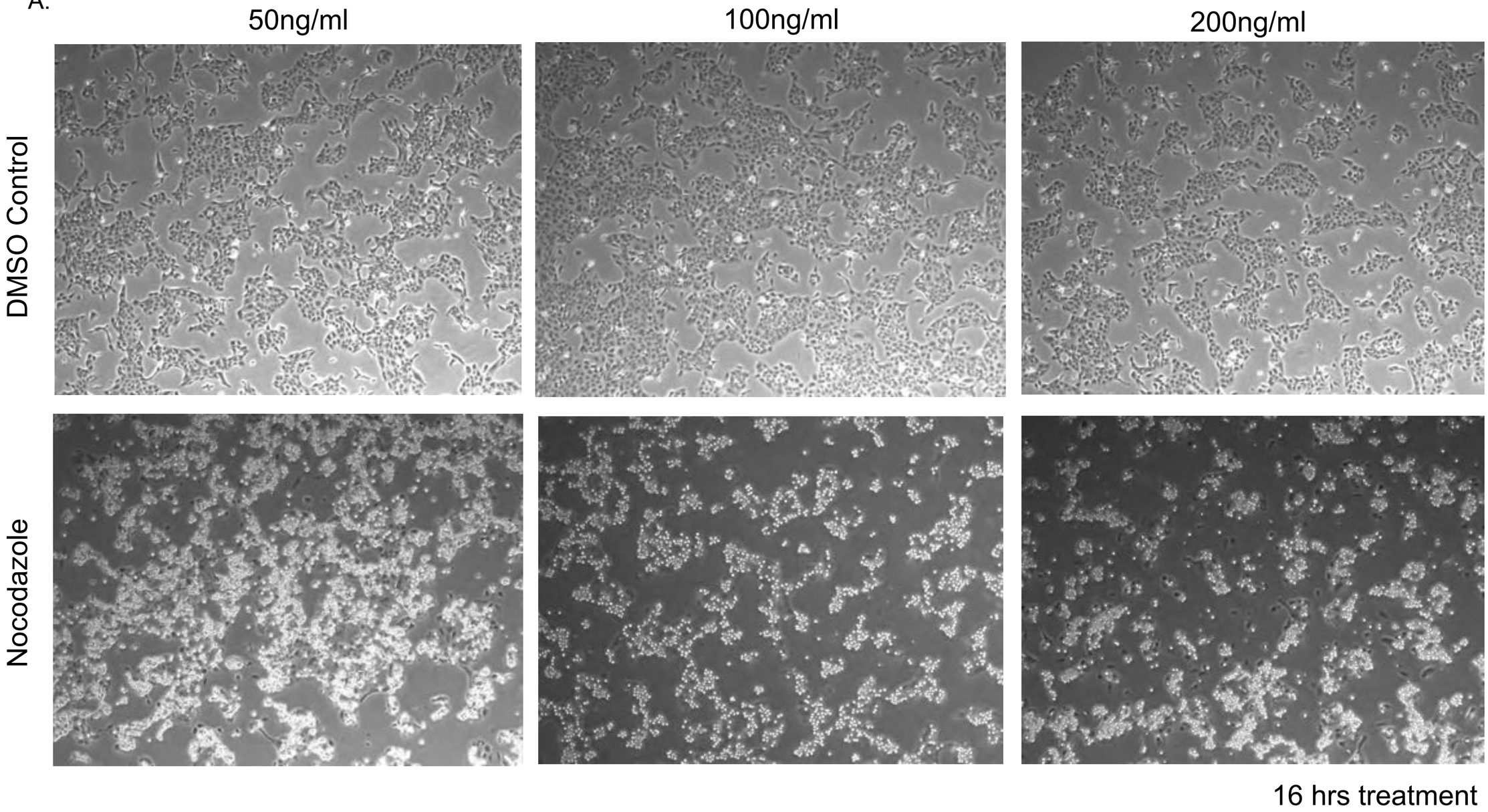


Suppl. Fig. 1

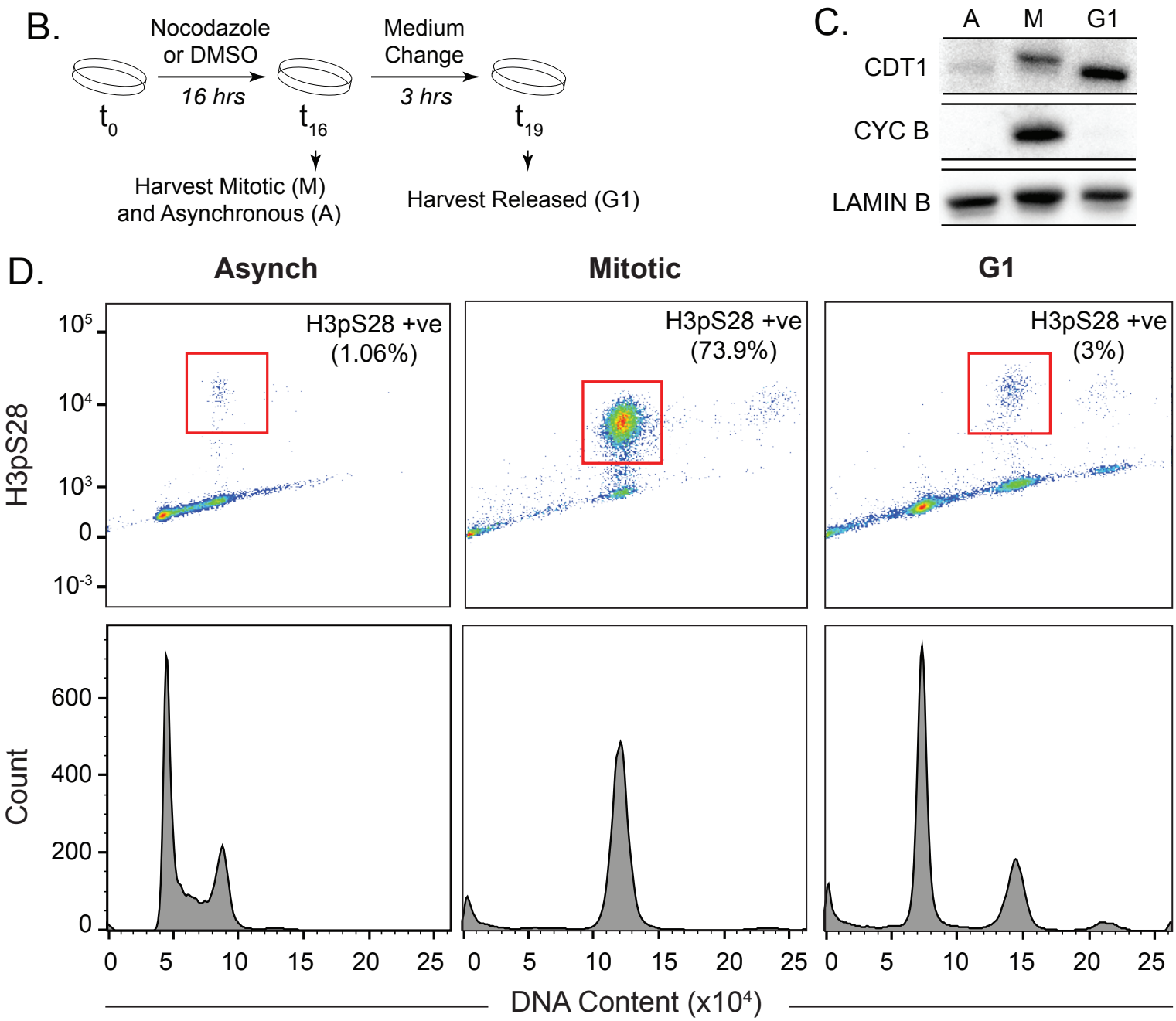

Prophase
Metaphase
Anaphase
Telophase
EGFP-RUNX1
HOECHST
MERGED

Suppl. Fig. 2

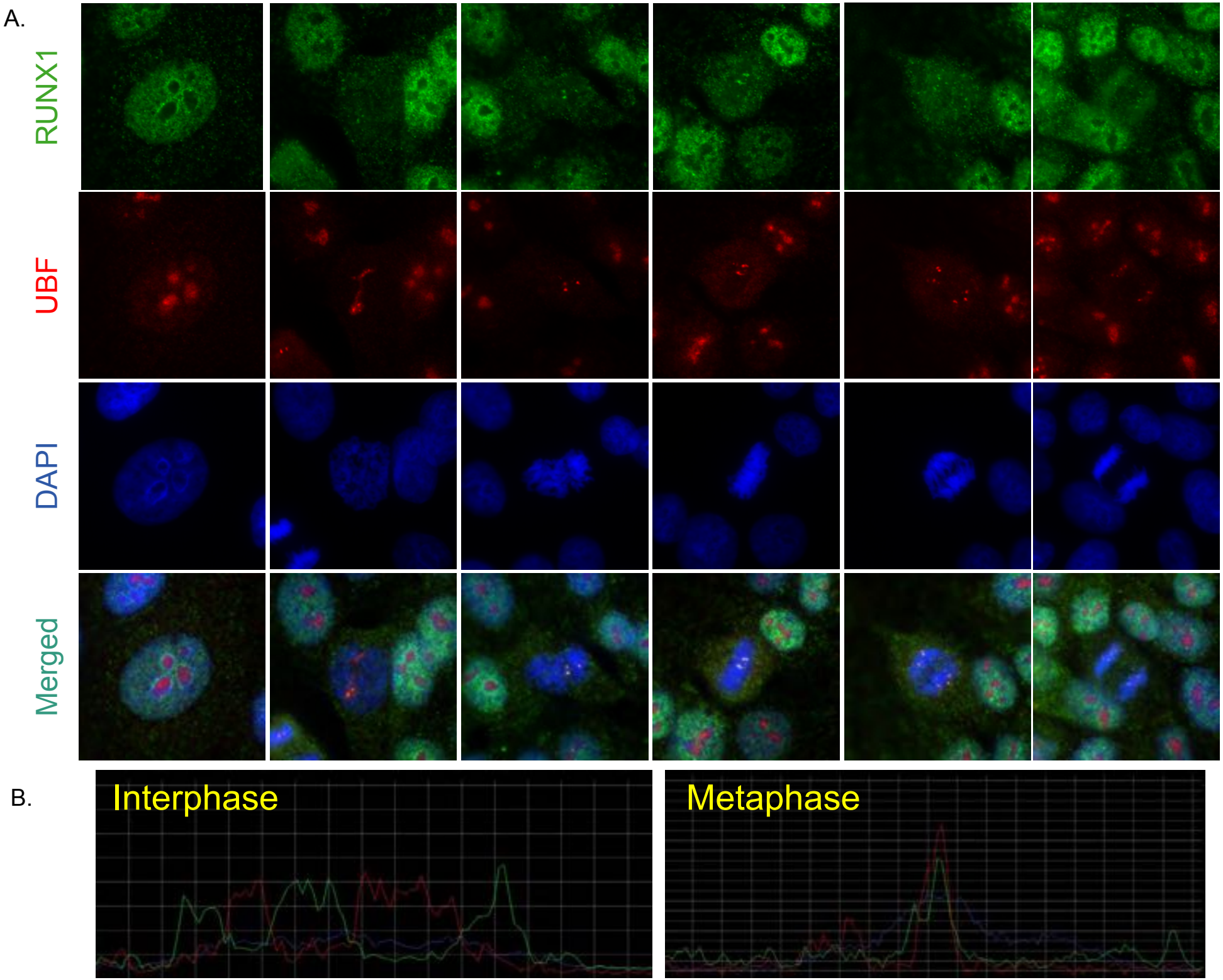
A.


50ng/ml
100ng/ml
200ng/ml
DMSO Control
Nocodazole
16 hrs treatment

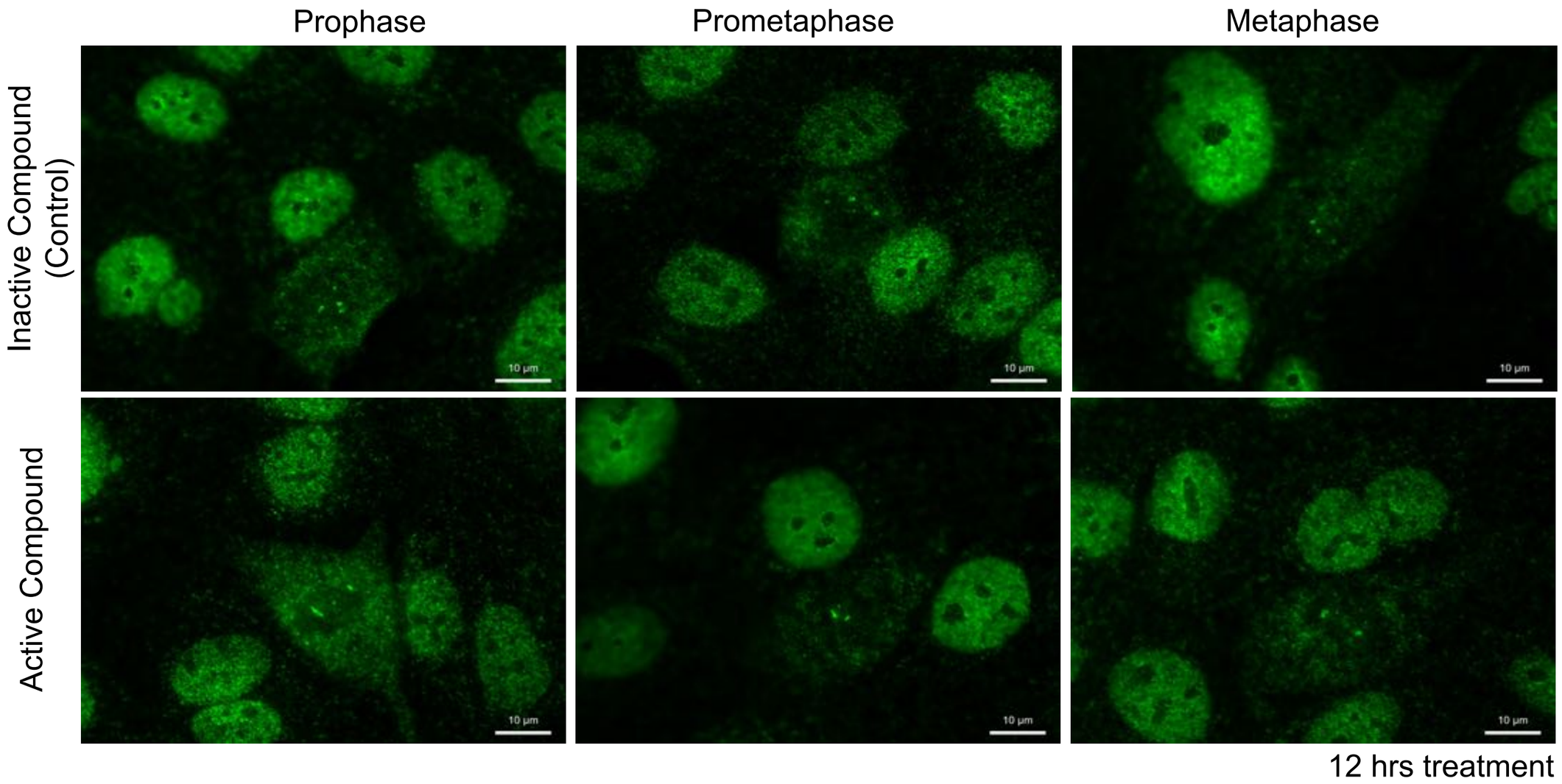
Suppl. Fig. 3


B.
Nocodazole
or DMSO
16 hrs
Medium
Change
3 hrs
t0
t16
t19
Harvest Mitotic (M)
and Asynchronous (A)
Harvest Released (G1)
C.
A
M
G1
CDT1
CYC B
LAMIN B
D.
Asynch
Mitotic
G1
H3pS28 +ve
(1.06%)
H3pS28 +ve
(73.9%)
H3pS28 +ve
(3%)
H3pS28
Count
DNA Content (x10⁴)

Suppl. Fig. 3



Suppl. Fig. 4


Prophase
Prometaphase
Metaphase
Inactive Compound (Control)
Active Compound
10 µm
12 hrs treatment

Suppl. Fig. 5