Transient telomerase inhibition alters cell cycle kinetics

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ABSTRACT

Telomerase is the ribonucleoprotein reverse transcriptase that catalyzes the synthesis of telomeres at the ends of linear human chromosomes and contributes to proper chromosomal capping function. Formation of the telomere-loop (T-loop), an obligate step before cell division can proceed, requires the generation of a 3'-overhang on the G-rich strand of telomeric DNA via telomerase or C-strand specific nucleases. Here, we discover telomerase activity is critical for efficient cell cycle progression using transient chemical inhibition by the telomerase inhibitor imetelstat. Telomerase inhibition caused changes in cell cycle kinetics and increased the proportion of cells in G2 phase, suggesting delayed clearance through this checkpoint. Investigating the possible contribution of unstructured telomere ends to these cell cycle distribution changes, we observed that imetelstat treatment induced γH2AX DNA damage foci in a subset of telomerase-positive cells but not telomerase-negative primary human fibroblasts. Chromatin-immunoprecipitation with γH2AX antibodies demonstrated imetelstat treatment-dependent enrichment of this DNA damage marker at telomeres. Notably, the effects of telomerase inhibition on cell cycle profile alterations were abrogated by pharmacological inhibition of the DNA-damage-repair transducer ATM. Additionally, imetelstat potentiation of etoposide, a DNA-damaging drug that acts preferentially during S/G2 phases of the cell cycle, also depended on functional ATM signaling. Our results suggest that telomerase inhibition delays the kinetics of T-loop formation in telomerase-positive cancer cells, resulting in the engagement of an ATM-dependent DNA-damage signal

that prevents cell cycle progression. This demonstrates for the first time that telomerase activity directly modulates the progression of the cell cycle through facilitation of T-loop formation.

SIGNIFICANCE STATEMENT

By forming higher-order capping structures, telomeres protect the ends of linear chromosomes from inappropriate recognition as DNA damage. An important component of this process is the formation of a 3'-overhang on the G-rich strand. This can be accomplished by the action of C-strand-specific nucleases or via telomere repeat synthesis by the telomere-maintenance enzyme telomerase. Here, using a chemical telomerase inhibitor, we demonstrate that telomerase facilitates the kinetics of telomere cap formation and passage through G2 phase. Inhibition of telomerase activity results in a prolonged ATM-dependent DNA-damage signal that alters cell cycle kinetics. Our data provide the biological rationale for exploring clinical telomerase inhibition strategies that leverage possible telomere-length-independent mechanisms of activity.

117 **INTRODUCTION**

118 Telomeres are nucleoprotein structures present at the ends of eukaryotic
119 linear chromosomes. Telomeres differentiate the ends of chromosomes from
120 random DNA breaks through the formation of capping structures called telomere
121 loops (T-loops). These higher-order capping structures are essential for proper
122 telomere function and cell cycle progression. In mammals, telomeres are
123 composed of repeated sequences of $(TTAGGG)_n$ nucleotides, the
124 complementary DNA strands, and associated proteins (1). Telomeres prevent the
125 loss of coding DNA sequence by buffering the lagging strand gap left by the
126 removal of RNA primers, thereby solving the “end replication problem”. Telomeric
127 DNA loss also results from T-loop resolution, an obligatory step to provide
128 access for DNA replication machinery. Together, these processes contribute to
129 the loss of 50-100 base pairs of 3’ terminal telomeric DNA with each replication
130 cycle (2). This telomere attrition forms the basis of the Hayflick Limit and restricts
131 the number of times a cell lineage may proliferate (3).

132 Telomeres are maintained by the ribonucleoprotein telomerase.
133 Telomerase has two main components: the catalytic telomerase reverse
134 transcriptase (TERT) and the template telomerase RNA (TER). TERT catalyzes
135 the synthesis of the hexanucleotide repeats by reverse transcribing the RNA
136 template sequences encoded in TER (4-7). This telomerase-mediated telomere
137 elongation promotes immortal growth by decoupling the cell division limit from
138 telomere length attrition.

139 TERT expression is normally repressed or only transiently activated in
140 somatic human cells, but telomerase activation and over-expression is detected
141 in 85-90% of human cancers (8,9). These differences make telomerase inhibition
142 an attractive therapeutic target (1,5-7,11,12,19). One current strategy for
143 telomerase inhibition is the drug imetelstat (GRN163L), a synthetic, lipid-
144 conjugated, 13-mer oligonucleotide N3' P5' thiophosphoramidate complementary
145 to the template of the TER component of telomerase (4,10,12,16-18,20). As a
146 competitive antagonist, imetelstat blocks the normal association of TER with
147 chromosomal ends (substrates), efficiently inhibiting *de novo* telomeric repeat
148 synthesis.
149 During the DNA-synthesis phase of the cell cycle, the activities of multiple
150 RecQ helicases, exonucleases, homologous recombination pathway effectors,
151 and histone methylation enzymes are coordinated to allow DNA polymerase and
152 telomerase (when expressed) to copy through T-loops and then restore the
153 heterochromatin state of newly replicated telomeres (8,10-14). Defects in
154 telomere-associated proteins involved in T-loop formation, such as helicases
155 (BLM, RTEL1), nucleases (Apollo), or telomere factors (TRF1, TRF2), have been
156 connected to uncapped telomeres, which can lead to cell cycle arrest and
157 ultimately, cell senescence or apoptosis (10,12,15-18,22). Additionally, the
158 correct rebuilding of chromosomal-end structures prior to cell division has been
159 shown to involve the Ataxia Telangiectasia Mutated (ATM) signal transduction
160 pathway (5-7,20,21). Activation of ATM and its associated PIKK-family member,

161 the ATM-related (ATR) kinase, is concurrent with T-loop resolution and the
162 resulting chromosome termini exposure (8,9,11,12,19).
163 Specifically, T-loop formation requires the creation of telomeric G-rich
164 overhangs that invade the double-stranded telomeric DNA region and form
165 displacement loops (1,10). In primary cells, telomerase is inactive and the
166 generation of G-rich overhangs and T-loops is dependent upon C-strand-specific
167 nuclease activity (1,10,12,15-18). In cancer cells, telomerase expression
168 provides another avenue for the de novo synthesis of G-rich telomeric DNA
169 repeats, in addition to nuclease activity (1,10,12,15-18). However, this role of
170 active telomerase in the kinetics of T-loop formation has been inferred from its
171 ability to synthesize G-rich telomeric repeats but has not been demonstrated
172 experimentally.
173 Here, we used imetelstat-induced chemical inhibition of telomerase activity
174 to explore the role of *de novo* telomeric DNA repeat synthesis in the kinetics of T-
175 loop formation. Since chemical inhibition is transient, we were able to study the
176 effects of telomerase activity on structure without significant shortening of
177 telomere length. Transient chemical inhibition also allows the immediate
178 observation of treatment effects, thus reducing the impact of compensatory
179 mechanisms that may confound observations.
180

181 **RESULTS**

182 ***Telomerase inhibition increases the proportion of cells in G2/M phases of***
183 ***the cell cycle***

184 During normal DNA replication, DNA-damage-response (DDR) signaling is
185 found transiently in late S/G2 phases at unstructured, open chromosome ends.
186 These signals have been detected in the forms of ATM-MRN and ATR-ATRIP
187 complexes (5,8,21,25,26). In order for cells to properly divide, DDR signals must
188 first be cleared by the reformation of T-loops. To understand how telomerase
189 may affect the kinetics of T-loop formation, we measured the effects of
190 imetelstat-induced telomerase inhibition on cell cycle progression.
191 Cells were treated with 10 μM inhibitory doses of imetelstat (5) or its
192 mismatch oligo control (MM) (27) for 24 h before harvesting and staining with
193 propidium iodide (PI) for cell cycle profiling using fluorescence-activated cell
194 sorting (FACS). In telomerase-positive mammary adenocarcinoma (MCF-7 and
195 MDA-MB 231) and colorectal carcinoma (HT29 and LS180) cells, a significant
196 increase in cell populations with 4N DNA content following imetelstat but not MM
197 treatment was observed (Fig. 1 a-d). In contrast, the cell cycle profiles of
198 telomerase-negative transformed cells (VA-13), which maintain telomeres via the
199 alternative lengthening of telomere (ALT) mechanism (28), and primary human
200 foreskin fibroblasts (BJ) were unaltered by both imetelstat and MM (Fig. 1 e-f).
201 The increased 4N cell population was likely due to a delay in exit from G2
202 phase rather than a cell cycle arrest, as previous studies have reported normal
203 cell growth following the removal of imetelstat (5). This suggests that treated cells
204 can return to normal cycling after the removal of drugs. To confirm the delayed
205 exit from G2 phase, we further investigated the proliferative capacity of cells after
206 imetelstat treatment. We used the IncuCyte Zoom live cell imaging system and

207 red fluorescent NucLight-tagged MDA-MB 231 cells to measure the effects of
208 continuous imetelstat treatment on cell proliferation over 7 days. Consistent with
209 a small but accumulative growth disadvantage conferred by delayed clearance
210 from G2 phase, we observed lower nuclear counts in cells exposed to 10 μM
211 imetelstat compared to untreated cells (Supplementary Fig. 1 a). A lower dose of
212 imetelstat (2 μM) resulted in an intermediate growth effect that is in agreement
213 with reduced inhibition of cellular telomerase activity (Supplementary Fig. 2, left
214 panel).

215 In order to measure growth in MCF-7 cells, we used the same treatment
216 regimen and measured absolute cell counts by Coulter counting. Similar to
217 before, a subtle growth defect was observed in MCF-7 cells treated with 10 μM
218 imetelstat (Supplementary Fig. 1 b). In contrast, imetelstat had no apparent effect
219 on cellular proliferation of telomerase-negative VA-13 cells (Supplementary Fig. 1
220 c). Telomerase activity in MDA-MB 231, MCF-7, and HT29 cells was effectively
221 inhibited by imetelstat in a dose-dependent manner, as confirmed by telomeric
222 repeat amplification protocol (TRAP) assays (Supplementary Fig. 2). These
223 results are consistent with a subtle imetelstat-induced defect in cell growth that is
224 revealed under longer-term incubation conditions. Together, our data suggest
225 that transient telomerase inhibition causes changes in cell cycle kinetics that
226 result in a growth disadvantage.

227

228 ***ATM inhibition abolishes the increase in 4N DNA cells caused by imetelstat***
229 ***treatment***

230 As ATM activation is concurrent with the resolution and rebuilding of telomere
231 structures, we next examined whether the effects of imetelstat on cell cycle
232 progression depend upon active ATM signaling. We applied pharmacological
233 inhibitor order-of-addition treatment regimens using imetelstat (Im) and the
234 specific ATM inhibitor KU55933 (Ku). The treatment conditions tested include:
235 single drug treatments of imetelstat (Im|Im) or KU55933 (Ku|Ku) for 48 h;
236 combinatorial treatment of imetelstat for 24 h then both imetelstat and KU55933
237 for another 24 h (Im|Im + Ku); and combinational treatment of KU55933 for 24 h
238 then both imetelstat and KU55933 for another 24 h (Ku|Im + Ku) (Fig. 2 a).
239 Consistent with Figure 1 a, imetelstat (Im|Im) treatment in MCF-7 cells
240 increased the population of cells with 4N DNA content relative to the population
241 distribution in untreated cells (Fig. 2 b). Notably, this effect on cell cycle
242 progression was abrogated by KU55933 pretreatment (Ku|Im + Ku) but not by
243 treatment with KU55933 after imetelstat exposure (Im|Im + Ku). Treatment with
244 ATM inhibitor alone (Ku|Ku) resulted in a reduction in the cell population with 4N
245 DNA content, but this reduction was not statistically significant in our data
246 analysis.
247 Treatment-induced cellular toxicities can also affect cell cycle profiles. To
248 differentiate this possibility from phase-specific cell cycle regulation, we analyzed
249 sub-G1 populations by FACS as a functional readout of cell death at the time of
250 DNA content analysis. The proportions of sub-G1 cell populations were not
251 significantly affected by single imetelstat (Im|Im) and KU55933 (Ku|Ku)
252 treatments, or by any treatment combinations, indicating that these changes in

253 cell cycle profiles were unlikely to be caused by increased rates of cell death
254 (Fig. 2 c).
255 Additionally, we assessed whether KU55933 interfered with the efficiency
256 of imetelstat-induced telomerase inhibition by measuring telomerase activity with
257 TRAP. Cells pre-treated with KU55933 (Ku|Im + Ku) also showed no telomerase
258 activity in the presence of imetelstat (Supplementary Fig. 3), confirming that ATM
259 inhibition did not affect imetelstat's ability to completely inhibit telomerase
260 actions. Together, these results imply that cell cycle alterations caused by
261 imetelstat-induced telomerase inhibition are dependent on functional ATM
262 signaling.
263
264 ***Imetelstat treatment induces telomere-specific DNA damage foci during the***
265 ***G2 phase of telomerase-positive cells***
266 In the absence of proper T-loop formation, uncapped telomeres are
267 recognized as DNA damage and accumulate DNA damage foci. The formation of
268 these damage foci is primarily mediated through the actions of ATM (26). To
269 confirm telomerase is involved in the normal kinetics of T-loop formation, we
270 examined the delayed clearance of ATM-induced DDR signals following
271 imetelstat treatment. Immunocytochemistry and confocal microscopy were used
272 to visualize the DDR marker γH2AX (Ser139), a standard marker of uncapped or
273 dysfunctional telomeres (1,20,21,25,26).
274 Imetelstat treatment over 24 h increased the number of cells with γH2AX
275 DDR foci in a subset of telomerase-positive MCF-7 and HT29 cells (Fig. 3 a-d)

276 but not telomerase-negative primary human BJ fibroblasts (Fig. 3 e-f). In parallel,
277 the mismatch oligo control had no significant effect on γH2AX DDR foci
278 accumulation. The observed increase in foci was not due to increased non-
279 specific background labeling in imetelstat-treated cells as illustrated by the
280 secondary antibody-alone controls (Supplementary Fig. 4).

281 To confirm the telomeric origin of imetelstat-induced γH2AX DDR foci, we
282 performed chromatin-immunoprecipitation using anti- γH2AX antibody. In
283 agreement with our immunocytochemistry observations, we confirmed increased
284 γH2AX ChIP signals following 24h imetelstat and etoposide treatments, but not
285 after treatments with the mismatch oligo, or vehicle control (Fig. 3 g-i).
286 Quantitative PCR measurements of γH2AX ChIP signals showed enrichment of
287 telomeric sequence following both imetelstat and etoposide treatments (Fig. 3 g).
288 In contrast, γH2AX signal enrichment at Alu-repeat sequence was only observed
289 with etoposide treatments (Fig. 3 h). When γH2AX signal enrichment at telomere
290 was normalized to the signals measured with the Alu-repeat reference, the
291 imetelstat group significantly different from the other three treatment groups,
292 suggesting that imetelstat induced the formation of telomere-specific γH2AX loci
293 (Fig. 3 i). This accumulation of γH2AX DDR foci at telomeres following imetelstat
294 treatment is consistent with an increase in uncapped telomeres caused by the
295 absence or inhibition of telomerase activity.

296 In order to assess the distribution of γH2AX DDR foci-positive cell
297 populations and their corresponding cell cycle phase, using
298 immunohistochemistry, we co-labeled imetelstat-treated MCF-7 and HT29 cells

299 with DDR and cell cycle phase markers. Since γH2AX DDR foci-positive cells co-
300 labeled with cytoplasmic cyclin B1, a marker of late S/G2 phases (Fig. 4 a-d), but
301 not phospho-histone H3 (H3P), an M phase marker in MCF-7 cells (Fig. 4 e-f),
302 the γH2AX DDR foci-positive cells were concluded to be residing in the S/G2
303 phases.

304 Consistent with the immunocytochemistry data, FACS measurements
305 showed the proportion of MCF-7 cells that were both cyclin B1-positive and
306 contained 4N DNA content increased with imetelstat treatment, relative to no
307 drug treatment and/or mismatch oligo controls (Fig. 4 g-h). Considering that the
308 magnitude of this increase was small, a delayed clearance from, rather than a
309 complete arrest at, G2 phase was more likely. Overall, our data indicate that
310 imetelstat-induced telomerase inhibition correlates with an accumulation of DDR
311 marker γH2AX and a delayed clearance of the telomere checkpoint at G2 phase
312 of the cell cycle.

313

314 ***Transient telomerase inhibition-induced DDR foci formation depends upon***
315 ***active ATM signaling***

316 Imetelstat was observed to delay G2 checkpoint clearance in an ATM-
317 activity dependent manner. To connect the role of ATM in G2 checkpoint
318 regulation with imetelstat-dependent γH2AX DDR foci formation, we again used
319 our order-of-addition experimental scheme coupled with immunofluorescent
320 detection of DDR signals. MCF-7 cells were treated with KU55933 and imetelstat
321 for 48 h, either alone or in different combinations, and γH2AX DDR foci were

322 quantified. As in our previous experiments, imetelstat treatment (Im|Im) resulted
323 in an increase in γH2AX DDR foci relative to the untreated (NT) negative control
324 (Fig. 5 a-b). Also as expected, we observed that treatment with KU55933 alone
325 (Ku|Ku) or before imetelstat addition (Ku|Im + Ku) abolished γH2AX DDR foci
326 formation. Interestingly, treatment with KU55933 after 24 h incubation with
327 imetelstat (Im|Im + Ku) only showed a slight, but not statistically significant,
328 reduction in γH2AX DDR foci formation. It is conceivable that blocking the activity
329 of ATM prevents the phosphorylation and propagation of new γH2AX foci but
330 does not remove the phosphorylated proteins that have already formed (Fig. 5 a-
331 b).

332 To provide further insight into the fate of imetelstat-treated cells that
333 escape G2-stalling, we labeled cells from different treatment groups for phospho-
334 histone H3P and quantified the proportion of cells progressing to M phase (Fig. 5
335 a, c). When no drug treatment was administered, ~5% of MCF-7 cells were H3P-
336 positive. Treatment with imetelstat alone (Im|Im) decreased the proportion of
337 cells in M phase, consistent with a stall at the G2 checkpoint due to persistent
338 DDR signaling. In contrast, cells treated with imetelstat and then KU55933 (Im|Im
339 + Ku) showed an increase in the proportion of cells in M phase, suggesting that
340 continuous ATM signaling is essential for the G2 stall caused by imetelstat-
341 induced DDR signaling. Blocking ATM signaling following transient telomerase
342 inhibition likely released MCF-7 cells from the G2 checkpoint and allowed
343 progression to the next phase of the cell cycle. This release of previously stalled
344 cells manifested as an increase in cells entering M phase.

345 Similar to continuous imetelstat treatment, treatment with KU55933 alone
346 (Ku|Ku) or prior to imetelstat addition (Ku|Im + Ku) reduced the number of M-
347 phase cells, suggesting that the order of ATM and telomerase inhibition is
348 important. Consistent with the relative decrease in 4N DNA cell populations
349 observed in our earlier FACS experiments (Fig. 2 b), ATM inhibition may induce
350 faster passage through G2/M phases using multiple parallel mechanisms (see
351 discussion) such that the effects of telomerase inhibition are masked. Our data
352 demonstrate that ATM inhibition before imetelstat treatment removed the effects
353 of telomerase-inhibition–induced G2 stalling, confirming that functional ATM
354 activity is essential for the telomere checkpoint.
355
356 ***Imetelstat potentiation of etoposide cytotoxicity depends upon active ATM***
357 ***signaling***

358 Previously, we observed that imetelstat potentiated the cytotoxicity of
359 S/G2-specific DNA-damaging agents, including the topoisomerase inhibitors
360 etoposide and irinotecan (5). Addition of ATM inhibitor KU55933 following 24 h of
361 imetelstat treatment (Im|Im + Ku) further increased the cytotoxicity of etoposide.
362 However, the cytotoxic effects from inhibition of ATM signaling before imetelstat
363 treatment (Ku|Im + Ku) were not tested in this previous study. To clarify the role
364 of ATM signaling in imetelstat-induced potentiation of etoposide cytotoxicity, we
365 again performed order-of-addition treatment experiments using the colony
366 forming unit assay (5-7,12,16). MCF-7 cells were pre-treated for 24 h with
367 imetelstat or KU55933 before the addition of etoposide with continued inhibitor

368 treatment or in combination with both imetelstat and KU55933 for 24 h. After 48 h
369 of treatment, cells were harvested and set in soft agar medium to recover for 2
370 weeks (Fig. 6 a).

371 In agreement with previous results, imetelstat (Im|Im) or KU55933 (Ku|Ku)
372 treatment alone significantly potentiated etoposide cytotoxicity in MCF-7 cells
373 (Fig. 6 b-c) (5,10-14,29). ATM inhibition consistently resulted in greater
374 potentiation of etoposide cytotoxicity than imetelstat-induced telomerase
375 inhibition alone. This observation was attributed to the broader role of ATM in
376 signal transduction regulation of cellular responses to double-strand DNA
377 damage (30), in addition to its role in telomere maintenance and structural
378 homeostasis.

379 Consistent with our previous data, an additive effect on etoposide
380 cytotoxicity was observed when the cells were treated with both inhibitors in
381 combination following imetelstat pre-treatment (Im|Im + Ku). In contrast, the
382 additive effect was lost when cells were pre-treated with KU55933 before
383 imetelstat and KU55933 addition (Ku|Im +Ku). These observations suggest that
384 functional ATM-dependent DDR signaling is required for the potentiation of
385 etoposide cytotoxicity by telomerase inhibition.

386 Notably, the rank order of treatment conditions leading to increased
387 sensitivities towards etoposide mirrors the rank order of treatment conditions
388 leading to increased DDR foci accumulation observed in our previous FACS
389 experiments. The most cytotoxic treatment condition (Im|Im + Ku) was also
390 observed to increase the proportion of cells in M phase in our previous ICC

391 experiments. Based on these observations, we reasoned that the increased
392 cytotoxicity might be linked to increased mitotic errors caused by the premature
393 release of chromosomes with unstructured telomeres from the G2 checkpoint.
394 Consistent with improperly capped telomeres proceeding to mitosis, following
395 treatment (Im|Im + Ku), we observed an increase in micronuclei formation that
396 indicated mitotic defects and increased genomic instability (Fig. 6 d-e). This
397 increase in the number of cells with mitotic defects acts as an additional cytotoxic
398 mechanism in etoposide-treated MCF-7 cells.
399

400 **DISCUSSION**

401 ***TERT addiction manifests as a kinetic advantage in cell cycle progression***

402 The role of telomerase in telomeric chromatin formation is inferred through
403 its *de novo* telomere synthesis function but has never been directly
404 demonstrated. Our data suggest that telomerase improves the kinetics of G-rich
405 overhang formation and thus, facilitates efficient higher-order telomeric chromatin
406 formation and clearance from the G2 checkpoint.

407 In telomerase-positive cells, imetelstat blocks the access of reverse
408 transcriptase to its telomeric DNA substrate, thereby preventing *de novo*
409 synthesis of G-rich telomere repeats (10,12,15-18,20,31). Consequently, ATM-
410 mediated DDR signals at unstructured telomeres take longer to resolve. This
411 results in delayed passage through cell cycle checkpoints and a temporary
412 accumulation of cells in G2 phase.

413 The lack of telomerase action stalls but does not entirely arrest cells at G2
414 phase because G-rich overhangs for T-loop formation can still be produced,
415 albeit with reduced efficiency, through the actions of multiple nucleases
416 (11,12,16,19,32). In the absence of G-strand synthesis, Exo1 provides more
417 substantial resection for T-loop formation. However, nuclease recruitment and
418 processing may have slower kinetics than telomere repeat synthesis (10,15).
419 Therefore, the inhibition of telomerase will delay, but not stop, the progression of
420 the cell cycle.

421 The observation that telomerase improves the kinetics of T-loop formation
422 is consistent with previous work from our laboratory showing that overexpression
423 of telomerase in telomerase-negative ALT cells conferred a growth advantage
424 and faster passage through S/G2 phases of the cell cycle (20,21,30,33). This
425 selective growth advantage may cause cancer cells to become “addicted” to
426 telomerase activity and explain the preponderance of telomerase overexpression
427 (>85%) for telomere maintenance over the alternate lengthening of telomere
428 mechanism in surveyed cancers (28).

429 As telomerase-negative cells reform T-loops using C-strand-specific
430 nucleases, their cell cycle progression is predicted to be unaltered by imetelstat
431 treatment (5,7,8,15,21,25,26). This prediction is supported by the lack of
432 significant decreases in cell growth in imetelstat-treated telomerase-negative cell
433 lines (Supplementary Fig. 1 c). Therefore, somatic tissues with low telomerase
434 expression are also predicted to have tolerable clinical toxicity profiles for
435 telomerase inhibition by imetelstat (1,5-7,20).

436 ***ATM signaling is necessary for telomerase actions at the T-loop***

437 Functional ATM is required for normal elongation of telomeres by
438 telomerase in human cells (5,10-14,23,24,34). ATM inhibition prior to imetelstat
439 treatment abolishes the delay in cell cycle progression induced by telomerase
440 inhibition. This observation may be due to the inhibition of telomerase recruitment
441 to the telomere in the absence of ATM activity (22) or the inhibition of a
442 telomerase-independent ATM function that leads to changes in G2 checkpoint
443 engagement (35).

444 Notably, ATM inhibition was previously shown to accelerate passage
445 through G2 phase by the removal of inhibitory phosphorylation of C-strand-
446 specific Exo1 nuclease (31). Increased Exo1 activity leads to faster G-rich
447 overhang formation and thus, increases the kinetics of T-loop formation in the
448 absence of telomerase actions. Accordingly, accelerated G2/M progression is
449 consistent with the observed trend of reduced populations of cells with 4N DNA
450 content following KU55933 treatment (Fig. 2 b).

451 Interestingly, reversal of the order of inhibitor addition, with the addition of
452 imetelstat before ATM inhibition, resulted in increases in M-phase cell
453 populations. This suggests that the ATM-dependent telomere checkpoint was
454 quickly engaged following telomerase inhibition. Subsequent ATM inhibition
455 released previously stalled cells from G2 phase to M phase, confirming that
456 continuous ATM signaling is crucial for the maintenance of this checkpoint (36).
457 In M phase, uncapped chromosome ends resulted in abnormal cell division
458 and/or mitotic catastrophe (Fig. 6 d-e). The higher levels of micronuclei formation

459 indicate possible mitotic defects and chromosomal instability due to errors in
460 telomere processing.
461
462 ***Therapeutic implications of short-term telomerase inhibition***
463 Previous work in our laboratory demonstrated that combining telomerase
464 inhibition by imetelstat with genotoxic agents potentiated the cytotoxic effects of
465 G2-specific DNA-damaging agents such as topoisomerase inhibitors (5,11,12).
466 Data from our current study indicate that this potentiating effect is likely due to an
467 ATM-dependent DNA-damage signal induced by telomerase inhibition. The
468 sustained DDR signal at unstructured chromosome ends may act in an additive
469 manner with G2-specific DNA-damaging agents such that a greater proportion of
470 cells pass the apoptotic threshold. Our hypothesis is supported by previous
471 observations that telomerase depletion in yeast caused chronic replication stress
472 and stalled replication forks (32). In this context, topoisomerase inhibitors and
473 other drugs that cause replicative stress may synergize particularly well with
474 imetelstat.
475 The potentiation of topoisomerase inhibitors by imetelstat may also be due
476 to pharmacodynamic interactions between telomerase inhibition and
477 topoisomerase inhibition. As previously observed, proper T-loop formation may
478 involve topoisomerase activity (7). The loss of telomerase activity in combination
479 with the absence of topology relief may further damage telomere structures and
480 result in cytotoxicity responses, thus providing another mechanism by which
481 imetelstat can potentiate topoisomerase inhibitor cytotoxicity.

482 In order for telomeres to shorten significantly, multiple rounds of cellular
483 replication are required. The long lag time associated with telomere shortening
484 has been a major theoretical barrier to the utilization of telomerase inhibitors for
485 anti-cancer chemotherapy (1). Recent clinical trials of imetelstat in myelofibrosis
486 and thrombocythemia have cast doubt on this premise. Telomere length did not
487 change in response to therapy and baseline telomere length was not predictive of
488 a positive therapeutic response (8,23,37). In this context, our data provide an
489 alternate explanation for the observed clinical effects of imetelstat: telomerase
490 inhibition in these hematopoietic cell types may induce distortions in cell cycle
491 kinetics without parallel observable effects on telomere-length regulation.
492 Telomeric-DNA-replication stress due to telomerase inhibition may be
493 partially relieved by an increase in the dNTP (purine) pool, as suggested in
494 previous studies (6,7,32). However, hematopoietic cancers frequently display
495 dysregulated dNTP metabolism (10,11,13,14,38). Therefore, this model predicts
496 that existing therapeutic agents targeting the available dNTP pools, such as
497 mycophenolic acids, will have synergistic effects with imetelstat treatments in
498 vulnerable hematopoietic cell types.
499 We observed that transient telomerase inhibition by imetelstat altered the
500 kinetics of T-loop formation through the engagement of the G2 checkpoint.
501 Imetelstat also sensitized telomerase-positive cells to G2-specific DNA-damaging
502 agents through delayed resolution of an ATM-dependent DNA-damage signal.
503 These observations allude to a separate mechanism by which telomerase
504 inhibition could affect telomere-maintenance kinetics and homeostasis. Our data

505 is relevant for understanding the role of telomerase in the formation of higher-
506 order telomeric-chromatin structures and cell cycle progression, thereby
507 presenting new testable hypotheses and possibilities for combination drug
508 regimens.
509

510 **MATERIALS AND METHODS**

511 **Cell lines and reagents**

512 MCF-7, MDA-MB 231, HT29, LS180, BJ fibroblasts, and WI-38 VA-13
513 were obtained from the American Type Culture Collection (ATCC). MDA-MB 231
514 (NucLight Red) cells were obtained from Essen Bioscience. Cell culture media,
515 antibiotics, and other cell culture reagents were purchased from Invitrogen/Life
516 Technologies unless otherwise noted. Cells were maintained under standard
517 culture conditions of 37°C and 5% CO_2 with penicillin and streptomycin antibiotics
518 (100 U each) and in the presence of appropriate fetal bovine serum (FBS)
519 concentrations (5-15%), as indicated by the ATCC.

520 Etoposide (Sigma/Aldrich) was administered in dose-response treatments
521 in 2-fold serial dilution and a maximum dose of 10uM. ATM inhibitor KU55933
522 (Calbiochem) was administered as 10 μM in DMSO, a concentration previously
523 determined to efficiently inhibit ATM function (5,21,25). Imetelstat and its
524 mismatch oligo control (MM) were obtained from Geron, resuspended in PBS,
525 and stored at -20°C. Stock concentrations were determined before each
526 experiment using UV-spectrophotometer absorbance. Imetelstat and MM were

527 administered at 10 μM, a dose previously determined to inhibit telomerase
528 activity by >100 fold in multiple cancer cell types (5).
529
530 **Colony forming unit assay (CFU)**
531 The assay was performed as previously described (1,5). Cells were seeded and
532 allowed to grow into individual colonies at 37°C under 5% CO_2 for 2 weeks.
533 Colonies were counted as positive if colony sizes exceeded 50 μm. Dose-
534 response analysis was performed using GraphPad Prism (v6.0b).
535
536 **Immunocytochemistry (ICC)**
537 Labeling was conducted as previously described (5,23,24). Primary antibodies
538 were sourced and diluted as follows: anti-phospho-histone H2AX (Ser139) 1:500
539 (JBW301 EMD Millipore), phospho-H3 1:500 (06-570 EMD Millipore), and cyclin
540 B1 1:100 (Santa Cruz H-433). Images were collected using a Zeiss LSM 700
541 confocal microscope and Zen 2012 (Zeiss) software. To quantify DNA-damage
542 response foci, the collected images were blinded and scored with >400 cells
543 analyzed for each condition. Nuclear abnormality scoring was conducted as
544 previously described (39).
545
546 **Fluorescence-activated cell sorting (FACS)**
547 Cells were plated and allowed to settle before treatment for 24 to 48 h with the
548 described drug regimens. The treated cells were harvested by trypsinization and
549 fixed with ethanol. FITC-Anti-Cyclin B1 (BD Biosciences) immunolabeling and

550 propidium iodide nucleic acid labeling were conducted according to the
551 manufacturer's protocol (Becton Dickinson). RNAse treatment was used to
552 remove non-specific signals. Labeled cells were sorted using a BD LSRII flow
553 cytometer (UBC Life Sciences Institute). For each cell sample, a minimum of
554 10,000 events were recorded. Flow Jo (Tree Star Inc) software and standard
555 gating procedures were used to quantify the sub-G1 population and the number
556 of cells with 2N and 4N DNA content.
557
558 **Chromatin-immunoprecipitation (ChIP)**
559 Cells were plated in 10 cm plates and allowed to settle before treatment for 24 h
560 with the described drug regimens. The treated cells were PBS-washed and
561 cross-linked in 1% formaldehyde. Cross-linked samples were collected by
562 scraping in ChIP lysis buffer (20 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM EDTA
563 pH 8.0, 1% Triton X-100, 0.1% SDS, 1X protease inhibitor cocktail) and
564 sonicated with a Covaris m220 ultrasonicator. The sonicated fractions were then
565 precleared with Protein G sepharose beads (GE Healthcare, #17-0618) and then
566 subjected to immunoprecipitation (IP) with 3 µg of anti-phospho-Histone H2A.X
567 (Millipore 05-636). IP with 30 µL of BSA-preblocked protein G beads (without
568 primary antibody) was used as control. Chromatin immunoprecipitates were
569 washed sequentially in ChIP Wash Buffer A (0.1% SDS, 1% Triton X-100, 2 mM
570 EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), ChIP Wash Buffer B (0.1% SDS,
571 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), ChIP
572 Wash Buffer C (0.25 M LiCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA,

573 10 mM Tris-HCl pH 8.0), and lastly TE buffer. The ChIP samples were reversed
574 cross-linked overnight in 65°C, and extracted DNA was purified with a DNA
575 cleanup kit (BioBasic). Quantitative PCR was performed using a Tel1b (telomere
576 sequence) primer set (Forward:
577 CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT, Reverse:
578 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT) and an Alu repeat
579 reference primer set (Forward: GACCATCCCGGCTAAAACG, Reverse:
580 CGGGTTCACGCCATTCTC) (40).
581
582 **Data analysis**
583 GraphPad Prism version 6 (GraphPad Software Inc, San Diego, CA) was used
584 for statistical analysis and data presentation. Student's *t*-tests or, where
585 appropriate, ANOVA followed by Fisher's LSD test were used to generate P-
586 values. $P < 0.05$ was considered statistically significant.
587
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594
595

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FIGURES

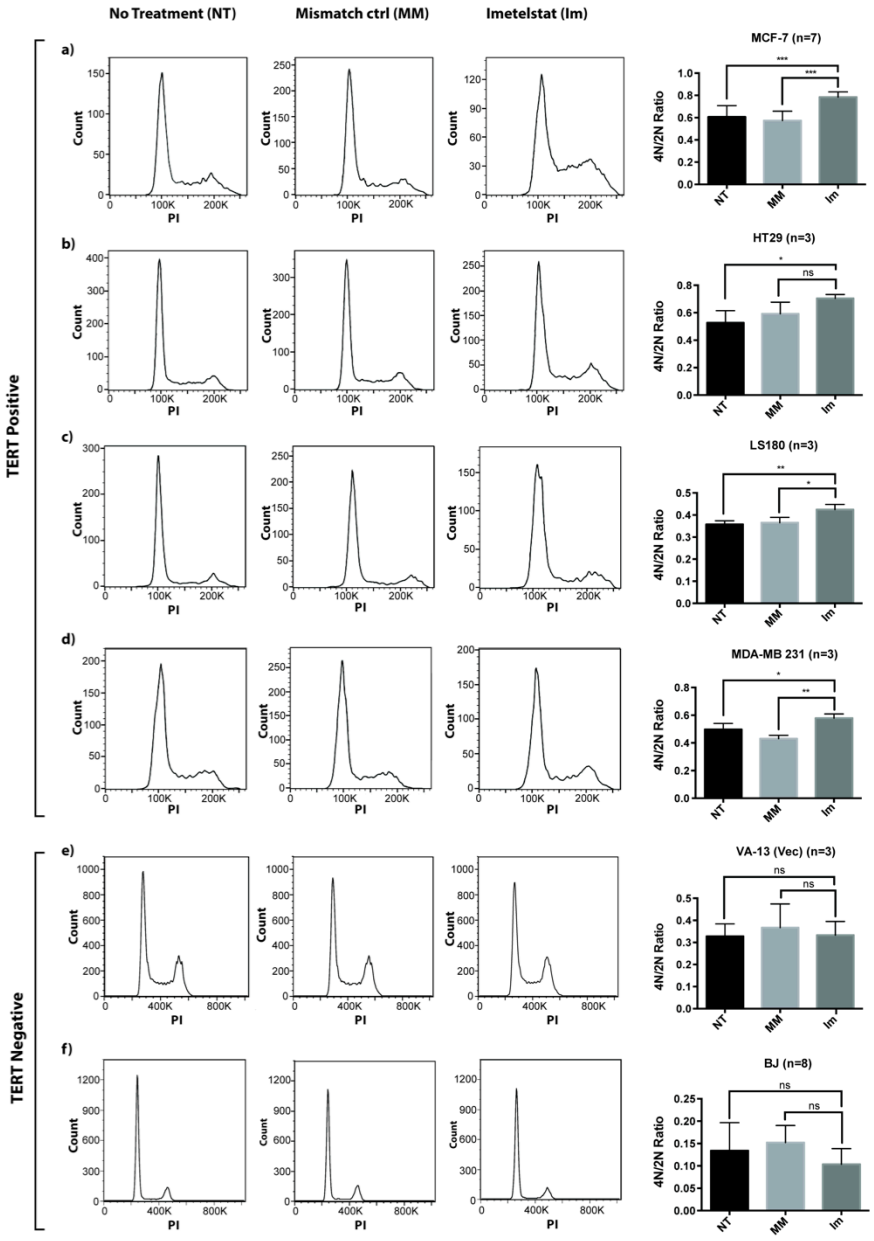

Figure 1. Telomerase inhibition increases the proportion of telomerase-positive cells with 4N DNA content. Cells were treated for 24 h with 10 μM imetelstat (Im) or its mismatch oligo control (MM). DNA content was analyzed using FACS and gating single cells by propidium iodide (PI) staining. Representative cell cycle profiles are shown for the indicated treatments and cell lines. (a-d) Imetelstat-induced telomerase inhibition increased the ratio of 4N to 2N cells in MCF-7, HT29, LS180, and MDA-MB 231 cell lines. (e-f) No change in ratio was observed in telomerase-negative VA-13 and BJ cell lines. ANOVA and Fisher's LSD test were used to generate P-values (* = P ≤0.05, ** = P ≤ 0.01, *** = P ≤ 0.001). Error bars represent SD.

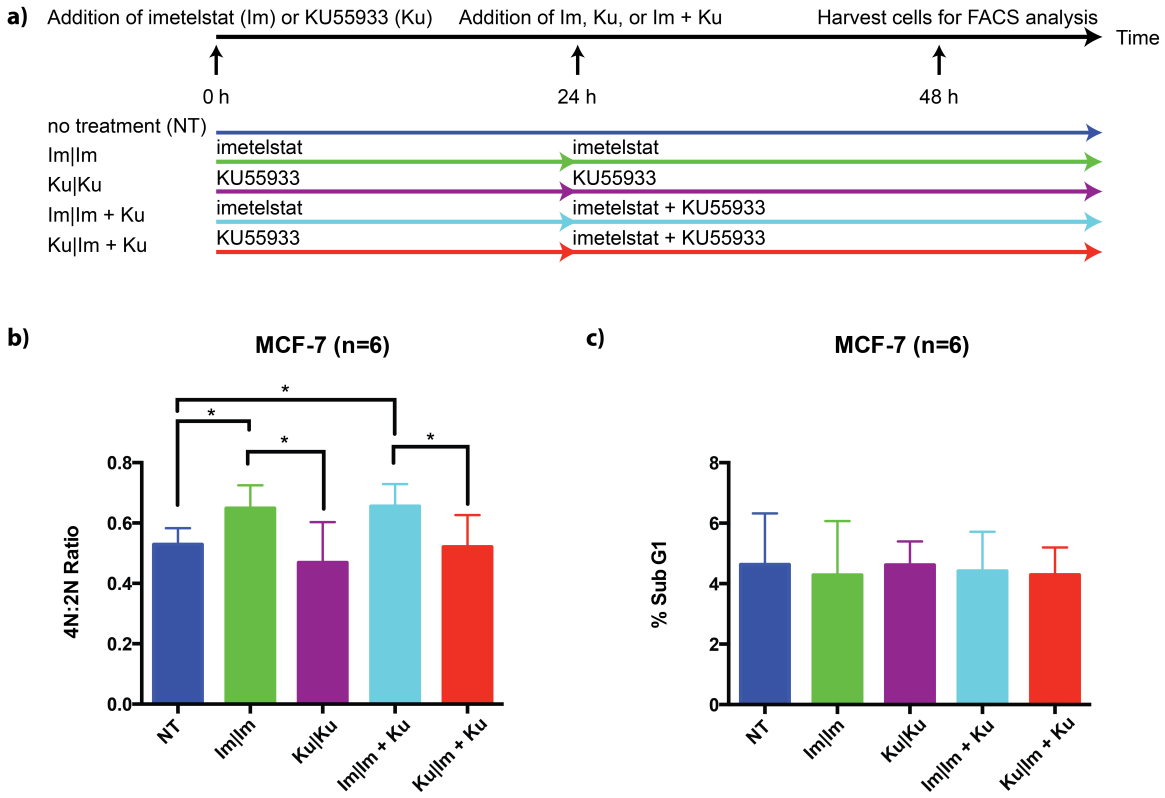

Figure 2. KU55933 and imetelstat affects cell cycle population distribution. (a) MCF-7 cells were treated with the indicated inhibitors (first 24 h | second 24 h) for 48 h. FACS analysis with propidium iodide staining was used to determine the 4N to 2N ratio. (b) Imetelstat (Im|Im) and imetelstat in combination with KU55933 (Im|Im + K) increased 4N DNA cell populations compared to no treatment (NT). Treatment with KU55933 in the first 24 h decreased 4N DNA cell populations compared to the respective treatment regimens using imetelstat in the first 24 h. (c) No difference in sub-G1 populations, a readout of apoptosis and cell death, was observed among cell lines undergoing different treatment regimens. ANOVA and Fisher's LSD test were used to generate P-values (* = P ≤0.05). Error bars represent SD.

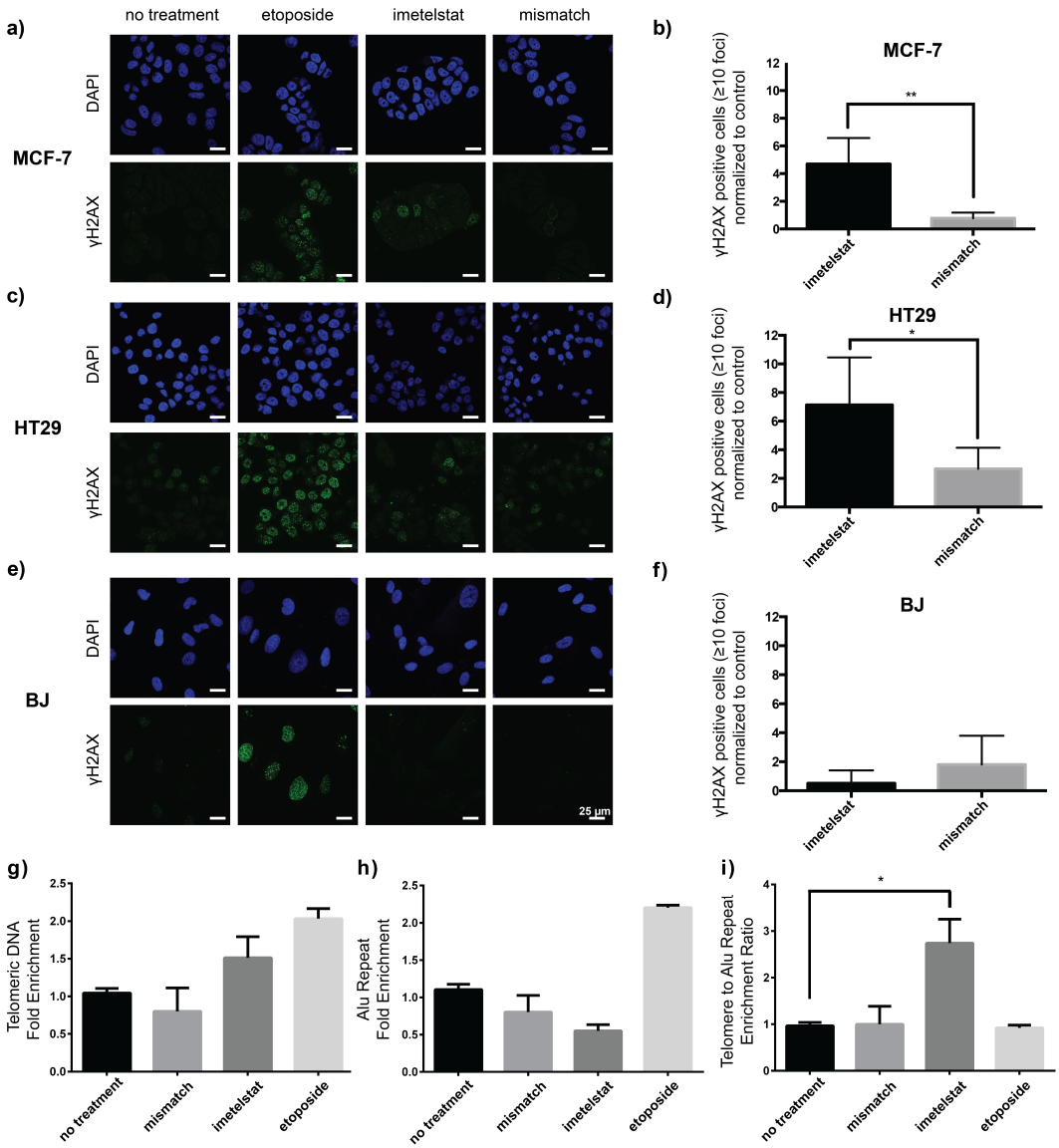

Figure 3. Telomerase inhibition induces DNA damage foci in telomerase-positive but not telomerase-negative cells. (a-d) Immunocytochemistry showed increased γH2AX DNA damage foci in a subset of telomerase-positive MCF-7 and HT29 cells treated for 24 h with imetelstat (10 µM). The mismatch control oligo (10 µM) did not induce this effect. Etoposide (1.4 µM) was used as a positive control. (e-f) Imetelstat did not induce DNA damage foci in telomerase-negative BJ primary human fibroblasts. Histograms show accumulation of cells with ≥10 foci, normalized to the numbers obtained from no treatment controls. Error bars represent SD. (g-i) γH2AX-ChIP-qPCR of (g) telomeric region and (h) Alu repeat region in MCF-7 cells. Enrichment values were normalized to beads only controls. (i) The ratio of ChIP enrichment of telomeric region over the Alu reference regions was indicative of telomere-specific enrichment of γH2AX signals (i). Error bars represent SEM. Student's *t*-test was used to generate P-values (* = P ≤0.05, ** = P ≤ 0.01, n ≥ 3).

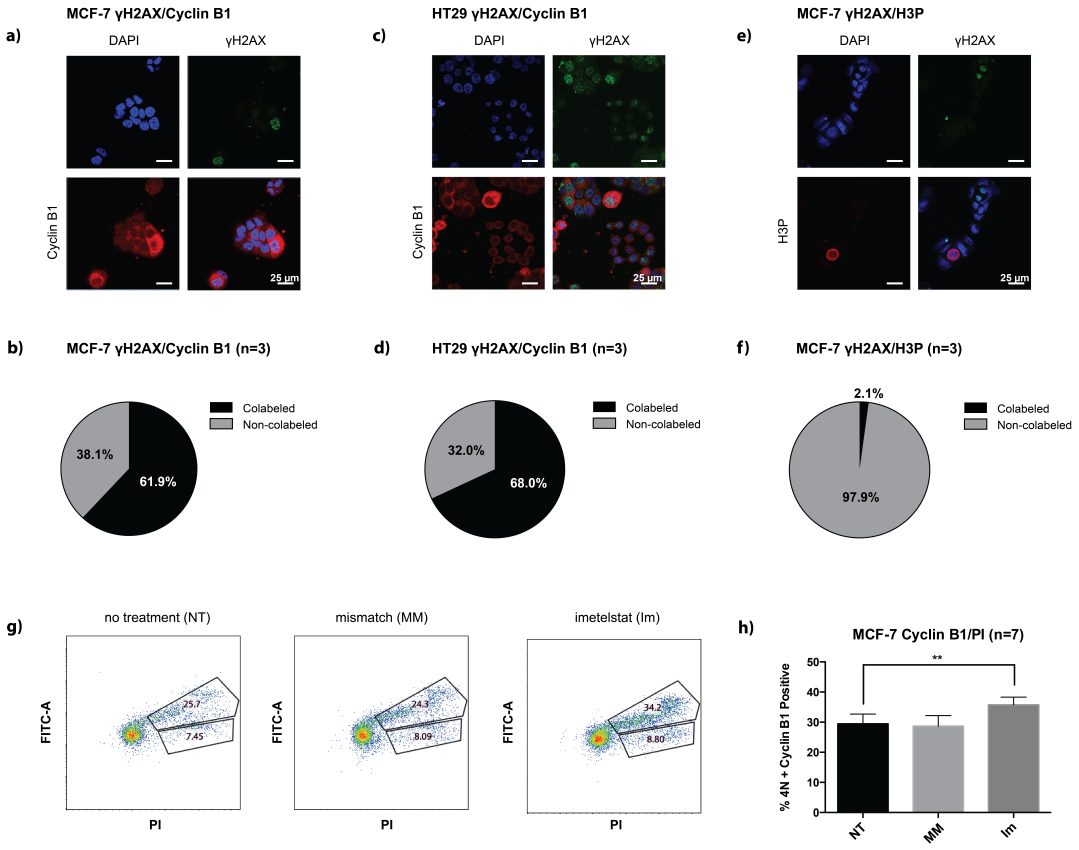

Figure 4. Imetelstat increases the population of cells in late S/G2 phases. (a-c) Following 24 h treatment with imetelstat (10 µM), MCF-7 and HT29 cells were labeled for γH2AX in combination with cytoplasmic cyclin B1, a marker of late S/G2 phases, or phospho-histone H3 (H3P), a marker of M phase. (d-f) DNA damage foci-positive cells (≥10 foci) co-labeled with cyclin B1 or H3P were quantified, with >400 cells analyzed for each condition. (g-h) MCF-7 cells were treated with imetelstat (Im), the mismatch oligo (MM) or no drug (NT) for 24 h then labeled with FITC-conjugated anti-cyclin B1 and propidium iodide (PI). ANOVA and Fisher's LSD test were used to generate P-values (* = P ≤0.05, ** = P ≤ 0.01). Error bars represent SD.

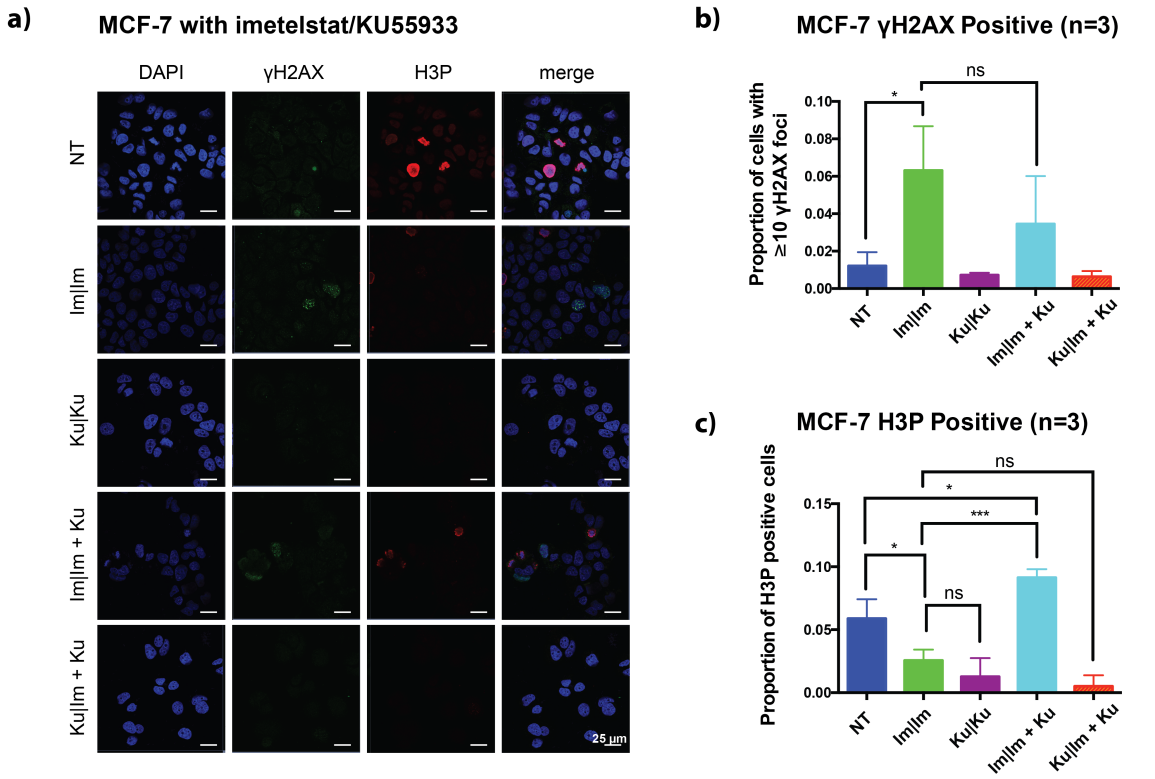

Figure 5. Functional ATM signaling is required for imetelstat-induced DNA damage foci formation and the G2/M checkpoint stall. (a) MCF-7 cells were treated with the indicated inhibitor(s) for 48 h using the same scheme as Fig. 2 before γH2AX and phospho-histone H3 (H3P) labeling. (b-c) Images were scored blindly to quantify co-labeling, with >400 cells scored per condition. ANOVA and Fisher's LSD test were used to generate P-values (* = P ≤0.05, ** = P ≤ 0.01, *** = P ≤ 0.001). Error bars represent SD.

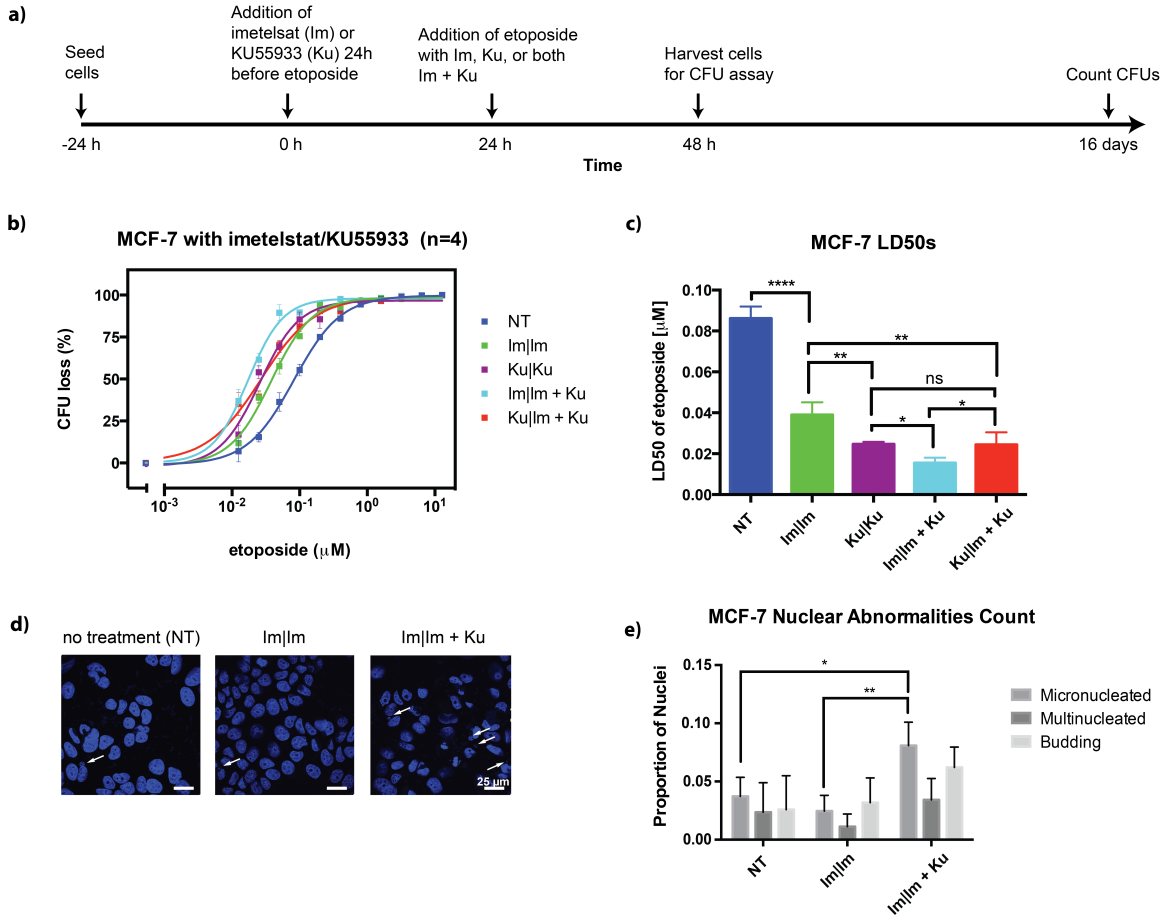

Figure 6. Imetelstat potentiation of etoposide cytotoxicity requires functional ATM signaling. (a) MCF-7 cells were treated with imetelstat (10 μM) or KU55933 (10 μM) for 24 h prior to addition of serial dilutions of etoposide (12.8 – 0.0125 μM) in combination with the previously used inhibitor or in combination with both inhibitors (Im + Ku). After 24 h incubation with etoposide, cells were counted and seeded in soft agar using the colony formation assay (CFU). Colonies were scored after 14 days. (b) Dose-response curves of MCF-7 cells given the indicated inhibitors (first 24 h | second 24 h in combination with etoposide) or no treatment (NT) before etoposide addition. (c) LD_{50}s obtained from dose-response curves. Error bars represent SEM. (d) Representative images of DAPI nuclear staining for treatments examined. Arrows indicate micronuclei. (e) Nuclear abnormalities were quantified from the same sets of images as in Fig. 5. Error bars represent SD. ANOVA and Fisher's LSD test were used to generate P-values (* = P ≤0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001).