1   **Release from cell cycle arrest with Cdk4/6 inhibitors generates highly synchronised cell**

2   **cycle progression in human cell culture**

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18   **Abstract**

19   Each approach used to synchronise cell cycle progression of human cell lines presents a

20   unique set of challenges. Induction synchrony with agents that transiently block progression

21   through key cell cycle stages are popular, but change stoichiometries of cell cycle regulators,

22   invoke compensatory changes in growth rate and, for DNA replication inhibitors, damage

23   DNA. The production, replacement, or manipulation of a target molecule must be

24   exceptionally rapid if the interpretation of phenotypes in the cycle under study are to

25   remain independent of impacts upon progression through the preceding cycle. We show

26   how these challenges are avoided by exploiting the ability of the Cdk4/6 inhibitors,

27   palbociclib, ribociclib and abemaciclib to arrest cell cycle progression at the natural control

28   point for cell cycle commitment: the restriction point. After previous work found no change

29   in the coupling of growth and division during recovery from CDK4/6 inhibition, we find high

30   degrees of synchrony in cell cycle progression. Although we validate CDK4/6 induction

31   synchronisation with hTERT-RPE-1, THP1 and H1299, it is effective in other lines and avoids

32   the DNA damage that accompanies synchronisation by thymidine block/release.

33   Competence to return to cycle after 72 hours arrest enables out of cycle target

34   induction/manipulation, without impacting upon preceding cycles.

35

36   **6 Keywords**

37   Restriction point, cell cycle synchronisation, Cdk4, palbociclib, thymidine block, cell cycle

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40   **Background**

41   Synchronised progression through the cell division cycle throughout a population supports

42   the ability to extrapolate the biochemical and functional attributes of the synchronised bulk

43   population back to infer behaviour in an individual cell [1,2]. Many approaches are popular.

44   Bulk levels of DNA or cell cycle markers support fractionation of live, or fixed, cell

45   populations into pools enriched for discrete cell cycle stages [3,4]. Although yields are low,

46   selection synchronisation based upon size, or mitotic shake off, are highly effective

47   approaches to isolate cells in one cycle phase from a large population of asynchronous cells

48   [5,6]. However, the ease of induction synchrony makes it the most widely applied approach.

49

50   Induction synchrony exploits the ability of transient exposure to a particular context to

51   accumulate cells at a discrete cell cycle stage, before removal of the context simultaneously

52   releases all cells in the population, to progress synchronously through subsequent phases of

53   the cell division cycle [1]. In yeasts transient ablation of cell cycle regulators through

54   reversible conditional mutations and the addition of mating pheromones predominate [7,8].

55   Although the advent of analogue sensitive versions of cell cycle kinases has introduced

56   analogous chemical genetic approaches into human tissue culture studies [9-12], induction

57   synchrony via serum starvation [13], or activation of either the DNA replication or spindle

58   assembly checkpoints [5,14] remain the most widely used.

59

60   The discovery that transient treatment with thymidine synchronised mitotic progression

61   [14], led to protocols that sharpened the degree of synchrony by imposing a second

62   thymidine block before releasing cells into the cycle of study [15-18]. This "double

63   thymidine block" remains one of the most popular choices, however, the power of this

64   approach, its reliance upon the DNA replication checkpoint to arrest S phase progression

65   with stalled DNA replication forks, comes at a cost. Although the cell cycle arrest is robust in

66   many lines, the stalled forks are prone to collapse over the extended arrest and subsequent

67   attempts at repair introduce damage and chromosomal rearrangements [19-21] . There are

68   also reports of understandable impacts upon RNA biology during the long block [22,23].

69   Thus, this popular approach can be of limited utility in the study of S phase, some

70   transcriptional and chromatin associated events.

71

72   When early cell cycle events are to be analysed, induction synchrony via release from a

73   mitotic arrest in the previous cell cycle, provides an attractive alternative. However, like

74   other forms of induction synchrony that rely upon an arrest within the cell division cycle,

75   prolonged cell cycle arrest will generate an imbalance in the many regulators, whose levels

76   fluctuate with cell cycle progression as a consequence of stage dependent transcription

77   and/or destruction [24]. Consequently, the next cycle may well be altered by excessive

78   regulatory activities, or substrates, inherited from the preceding, arrested, cycle. Incisive

79   studies by Ginzberg and colleagues revealed how counter-measures to accommodate some

80   imbalances promote adjustments in growth rates at two points in the cycle [25]. Prolonged

81   mitotic arrest can also initiate apoptotic pathways [26] and/or leave a memory of the mitotic

82   arrest that modifies cell cycle progression in the next cycle and beyond [27-30].

83

84   Thus, while highly informative for some questions, data obtained through traditional

85   induction synchrony approaches, that rely upon arrest *within* the cycle, have to be

86   interpreted with caution. They must be consolidated with complementary data from

87   alternative approaches, to reveal the commonalities that exclude the artefacts incurred in

88   one given approach to synchronisation.

89

90   A further challenge in synchronising cell cycle progression throughout a population arises

91   when there is a need to assess the impact of protein depletion, induction or replacement. It

92   is critical to ensure that the destruction, induction, or activation of a mutant variant starts

93   after the synchronising procedure is complete. If not, then the phenotype can be a legacy

94   arising from perturbation of progression through the previous cycle, rather than a direct

95   impact upon the cycle being studied. Advances in degron and PROTAC technologies may

96   overcome many of these challenges [31-33]. However, even with many induction

97   synchronisation approaches, the switch from one version of a protein to another must be

98   exceptionally rapid and complete if perturbation of the preceding cycle is to be avoided.

99

100   Inspired by the power of pheromone induction synchronisation at G1 phase of yeast cell

101   cycles [8,34], we explored the utility of induction synchrony with CDK4/6 inhibitors

102   palbociclib, ribociclib and abemaciclib. These inhibitors arrest cell cycle progression of

103   mammalian tissue culture cells at the restriction point in G1 phase prior to commitment to

104   the cell cycle. Synchronisation by induction from the natural pause point in the cycle

105   appeals because the cell cycle programme is yet to be set in motion and extended arrest via

106   Cdk4/6 inhibition does not invoke compensatory changes in cell cycle or growth controls;

107   rather it simply adjusts cell size control [25].

108

109   Cdk4 and Cdk6 kinases determine commitment to the cell cycle of many cells. They partner

110   Cyclin D and the Kip family members p21 and p27 to generate active trimeric kinase

111   complexes that phosphorylate the C terminus of the retinoblastoma (Rb) family proteins [35-

112   41]. This mono-phosphorylation supports further phosphorylation of Rb by Cdk1/Cdk2-Cyclin

113   E and Cdk1/Cdk2-Cyclin A complexes [10]. Hypo-phosphorylated Rb binds tightly to the

114   transcription factors of the E2F family, to block the transcription of genes required for cell

115   cycle commitment. Rb hyper-phosphorylation relieves this inhibition, to promote

116   transcription of cell cycle genes, including Cyclins E and A. Induction of these Cyclins rapidly

117   boosts Rb phosphorylation by Cyclin E and Cyclin A Cdk complexes [42]. Cyclin E and Cyclin A

118   complexes seal commitment to the cycle by inhibiting the anaphase promoting

119   complex/cyclosome (APC/C) activating component Cdh1, thereby stabilising APC/C^{Cdh1}

120   targets, including Cyclin A [43-47].

121

122   The key role played by Cdk4-Cyclin D and Cdk6-Cyclin D in presiding over cellular

123   proliferation, and the contrasting ability of mice to survive genetic ablation of Cdk4, Cdk6

124   and Cyclin D and the addiction of cancer lines to these kinases, prompted the development

125   of clinically successful Cdk4/6 inhibitors [48-52]. These inhibitors bind to the inactive Cdk4-

126   Cyclin D and Cdk6-Cyclin D dimers, rather than the active trimeric complexes, yet they

127   impose a very efficient cell cycle arrest [41]. It is thought that this counter-intuitive impact

128   arises from the sequestration of the inactive dimers, or monomeric kinases by the drugs.

129   This sequestration releases p21 and p27 to elevate concentrations of these Cdk2 inhibitors,

130   to a level where they block the ability of the Cdk2-Cyclin A and Cdk2-Cyclin E complexes to

131   promote the feedback loops and S phase events that drive commitment to the cycle [41,53].

132   Each drug has a specific off target profile. Ribociclib's impressive specificity is almost

133   matched by palbociclib, while abemaciclib shows significant off-target impacts, with notable

134   activities towards Cdk1, Cdk2, Cdk7 and Cdk9 complexes, that can induce an arrest in G2

135   alongside G1 [54]. Paradoxically, this off-target impact may account for abemaciclib's

136   greater efficacy in some clinical settings [54,55].

137

138   We describe how CDK4/6 induction synchrony generates highly synchronous progression

139   through the cell cycles of a number of lines, without the marked appearance of a marker of

140   DNA damage, foci of staining of phospho-γ-H2AX, that accompanies thymidine induction

141   synchronisation. The ability to return to cycle after 72 hours arrest in G1 with palbociclib

142   will support a broad range of manipulations in the arrested, non-cycling state [56]. Thus, any

143   impacts upon progression through the cycle of study will not be a secondary consequence of

144   perturbation of the preceding cell cycle.

145

146   **Methods**

147   **Cell lines and Cell Culture**

148   The following lines were purchased from ATCC at the start of the study: hTERT-RPE1 (ATCC

149   Cat# CRL-4000, RRID:CVCL_4388), A-549 (NCI-DTP Cat# A549, RRID:CVCL_0023), THP-1 (CLS

150   Cat# 300356/p804_THP-1, RRID:CVCL_0006) and NCI-H1299 cell line (NCI-DTP Cat# NCI-

151   H1299, RRID:CVCL_0060). Upon receipt, lines were expanded and frozen into aliquots of 1 x

152   10^7 cells that were expanded by at least two cycles of continual passage prior to each

153   experiment. Unless otherwise stated, hTERT-RPE-1 and A549 were maintained in high

154   glucose Dulbecco's modified eagles medium (DMEM: D6546, Sigma Aldrich) supplemented

155   with 10% FBS (Hyclone, South American Origin, SV30160.03, GE Healthcare) 2 mM

156   GlutaMAX (Gibco, 35050061) and 1000 U/ml Penicillin/Streptomycin (Gibco, 15140122).

157   THP1 and H1299 were maintained in Roswell Park Memorial Institute 1640 medium (RPMI)

158   supplemented with 2 mM GlutaMAX (61870044, Gibco), 10% FBS (Hyclone, South American

159   Origin, SV30160.03, GE Healthcare) and 1000 U/ml Penicillin/Streptomycin (Gibco,

160   15140122). Cells were maintained at a confluency of less than 70% and populations were

161   not passaged more than 10 times before any experiment. In Figure 2e, where hTERT-RPE-1

162   cells are grown in RPMI, cells were originally grown from the frozen stock in DMEM,

163   passaged twice in RPMI, then plated in RPMI at the start of the experiment.

164   **Drug treatment**

165   Palbociclib (PD-0332991), Abemaciclib (LY2835219) and Ribociclib (LEE011) were purchased

166   from Selleck (catalogue numbers S1116, S5716, S7440 respectively), while thymidine (T1895)

167   and nocodazole (M1404) were from Sigma-Aldrich. Palbociclib, Abemaciclib, Ribociclib and

168   Nocodazole were dissolved in DMSO to generate stock solution of 10 mM in each case.

169   Thymidine was dissolved in water to make a stock solution of 100 mM. All stocks were

170   stored in aliquots at -20 °C.

171   For adherent cell lines (RPE1, A549 and H1299), cells were released from the substrate by

172   treatment with trypsin (15400054, Gibco), pelleted by centrifugation at 300 g for 5 minutes

173   before resuspension in growth media. Cells were counted using a TC20 automated cell

174   counter (BioRad) and 2.5 x 10^5 cells were plated in a 10 cm dish (353003, Falcon) with 10 ml

175   media (plating density = 4.4 x 10^3 per cm^2). For the THP1 suspension line, cells were pelleted

176   by centrifugation at 300 g for 5 minutes, resuspended in growth media and counted, before

177   1 x 10^6 cells were seeded in 10 ml media in a 10 cm dish. Cells were then incubated for 6 or

178   12 hours (see figure legends) before drug was added.

179   **Flow Cytometry**

180   For cell cycle analysis, cells were washed once with phosphate buffered saline (PBS) before

181   releasing from the substrate with trypsin, and washed in phosphate buffered saline before

182   fixation in ice-cold 70% Ethanol and freezing at -20°C for at least 18 hours up to a maximum

183   of 2 weeks.   Fixed cells were pelleted, washed 3 times in PBS at room temperature before

184   50 μl 100 μg/ml RNase (NB-03-0161, Generon) was added to the final pellet, followed by 500

185   μl 50 μg/ml propidium iodide (P4170, Sigma-Aldrich) dissolved in PBS. Samples were

186   analysed within a window of between 30 minutes and 6 hours after addition of propidium

187   iodide. Data were acquired on a BD LSR II flow cytometer (BD Biosciences) using FACSDiva™

188   software (BD Biosciences) and analysed with FlowJo software (BD Biosciences). 1 x 10^4 cells

189   were counted for each sample.

190   S phase status was monitored by measuring 5-ethynyl-2'-deoxyuridine (EdU) incorporation

191   with the Click-iT™ Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (ThermoFisher,

192   C10632) according to the manufacturer's instructions. 1 μM EdU was added at the time of

193   release to monitor cumulative DNA content between the time of release and the point of

194   fixation. For staining, cells were trypsinised and washed with 3 mL of 1% BSA in PBS, then

195 incubated with 100 µl of Click-iT™ fixative for 15 minutes, pelleted, washed with 3 mL of 1%

196 BSA in PBS and left at 4 °C overnight. Cells were resuspended in 100 µL of 1X Click-iT™

197 permeabilization and wash reagent and incubated with 500 µl of Click-iT™ Plus reaction

198 cocktail for 30 minutes, before washing once with 3 mL of 1X Click-iT™ permeabilization and

199 wash reagent and resuspended in 500 µL of 1X Click-iT™ permeabilization and wash reagent.

200 FxCycle Violet stain (F10347, Invitrogen) was added to a final concentration of 1 µg/ml.

201 Samples were analysed on a BD LSR II flow cytometer (BD Biosciences) using FACSDiva

202 software (BD Biosciences) and analysed with FlowJo software (BD Biosciences). 10^4 cells

203 were counted per sample.   For quantification the peak of 2N cells was used to gate an

204 assessment of 2N DNA content, as the overlap between S phase and G2/M made it

205 challenging to categorically assign cell cycle status on the basis of DNA content alone. Thus,

206 throughout the manuscript we monitor cell cycle progression as a reduction in 2N DNA

207 content.

208 **Immunofluorescence**

209 Cells were grown on 13 mm coverslips (No. 1.5, VWR, 631-0150P) in 10 cm dishes (353003,

210 Falcon) using the appropriate growth conditions. For EdU staining, the Click-iT™ EdU Cell

211 Proliferation Kit for Imaging, Alexa Fluor™ 594 dye (Invitrogen, C10339) was used. 1 hour

212 prior to harvesting cells, 10 µM EdU was added to the growth media. Cells were washed in

213 PBS before fixing for 20 minutes in 2% PFA in PBS, before three washes in PBS + 0.1% Tween

214 and storage in PBS + 0.1% Tween at 4 °C overnight. Permeabilisation in PBS + 0.5% Triton-X-

215 100 was followed by 3 washes in PBS + 0.1% Tween. The Click-iT™ reaction for EdU

216 detection according to the manufacturer's instructions. Cells were washed 3 times in 5%

217 Bovine Serum Albumin (BSA; ThermoFisher Scientific, 11423164) in PBS then incubated with

218 primary antibody to H2AX (Ser139), clone JBW301 (Millipore Cat# 05-636, RRID:AB_309864)

219 at a dilution of 1 in 500 for 1 hour before 3 washes in PBS + 5% BSA and incubation with a 1

220 in 500 dilution of goat anti-mouse IgG Alexafluor 488 antibody, (Thermo Fisher Scientific

9

221   Cat# A32723, RRID:AB_2633275), and 2 mg/ml 4',6-diamidino-2-phenylindole (DAPI,

222   ThermoFisher Scientific, 11916621) for 1 hour. After a further 3 washes in PBS + 0.1%

223   Tween, coverslips were mounted by inversion onto 2 µl of Vectashield Antifade Mounting

224   Medium (Vector Labs H1000). Slides were analysed using an Axioskop2 (Zeiss, Inc)

225   microscope.

226   **Protein analysis**

227   To collect hTERT-RPE1 protein samples, culture medium was removed, by aspiration, from a

228   10cm plate, before two washes in PBS and addition of 400 µl TruPAGE LDS sample buffer

229   (Sigma-Aldrich, PCG-3009) containing complete protease inhibitor cocktail (Roche,

230   11697498001) PhoSTOP (Sigma-Aldrich, 4906837001) and DTT to the plate. Cells were

231   scraped from plate for transfer into a 1.5 ml microfuge tube and snap frozen in liquid

232   nitrogen for storage at -80°C. For THP1, cells were centrifuged at 300 g for 3 minutes and

233   the pellet resuspended in sample buffer as above, frozen in liquid nitrogen and stored at -

234   80°C. Samples were heated at 70°C for 10 minutes before loading on a 10 cm, 10% precast

235   TruPAGE gel (Sigma-Aldrich PCG2009) with TruPAGE SDS running buffer (Sigma Aldrich

236   PCG3001) and transferred to PVDF membrane (BioRad, 1620177) by wet transfer with

237   TruPAGE transfer buffer (Sigma-Aldrich PCG3011). Membranes were blocked in 5% milk and

238   incubated in 2% BSA with primary antibodies to GAPDH (Cell Signaling Technology Cat#

239   13084, RRID:AB_2713924), Eg5 (Sigma Aldrich Cat# SAB4501650, RRID:AB_10747045), Wee1

240   (Cell Signaling Technologies Cat# 13084, RRID:AB_2713924) or pHH3 ser10 (custom antibody

241   to peptide ARTKQTARKS*TGGKAPRKQLASK: Eurogentec) overnight at 4°C. After washing,

242   membranes were incubated for 1 hour at room temperature, with the appropriate

243   secondary antibody (Anti-mouse IgG phosphatase conjugated antibody (Sigma-Aldrich Cat#

244   A3688, RRID:AB_258106), or Anti-rabbit IgG phosphatase conjugated antibody (Sigma-

245   Aldrich Cat# A3687, RRID:AB_258103), washed and developed with chromogenic 5-Bromo-4-

246   chloro-3-indolyl phosphate (BCIP, Sigma-Aldrich, B6149).

247

248 **Results**

249 **Efficient synchronisation of hTERT-RPE1 cell cycle progression with transient palbociclib**

250 **treatment**

251 The telomerase immortalised hTERT-RPE1 cell line is widely used for cell cycle and mitotic

252 studies, yet is refractory to double thymidine block induction synchronisation and yields low

253 levels of synchrony following release from serum starvation. We therefore chose this

254 popular line to assess the efficiency of G1 arrest and release by transient exposure to

255 palbociclib as it would be a major benefit to develop synchronisation approaches in this line

256 [56,57].

257

258 Cells were grown to 1.5 x 10^4 cells per cm^2 in serum supplemented (10%) Dulbecco's

259 modified eagles medium (DMEM) before release from the substrate with trypsin and plating

260 at a density of 4.4 x 10^3 cells per cm^2. 6 hours later palbociclib was added in a range of

261 concentrations from 50 nM to 1 μM to two identical populations. 24 hours later one

262 population was fixed, while the palbociclib containing medium for the other was replaced

263 with pre-warmed medium containing 330 nM nocodazole, before this sample was fixed 24

264 hours later. DNA content assessment by fluorescence activated cell sorting (FACS) analysis of

265 propidium iodide (PI) stained samples revealed a tight arrest at the 24 hour time point with

266 2N DNA content at all palbociclib concentrations of 100 nM and higher (Figure 1a,b). Cells

267 readily released into a 4N arrest after being arrested in G1 for 24 h with 100 nM (Figure 1a)

268 and 200 nM palbociclib, however release was less efficient when the arrest was imposed by

269 500 nM and 1 μM palbociclib (Figure 1b) as the 2N DNA content remained high 24 h after

270 nocodazole addition.

271

272 Monitoring DNA content at hourly intervals after release from 150 nM palbociclib arrest

273 revealed a synchronous progression from G1 arrest with 2N DNA, through S phase into

274 G2/M phases with 4N DNA content before a return to 2N DNA at 20 hours (Figure 1 c,d). As

275 with all experiments presented herein, 24, or 48, hour periods were covered by sampling

276 parallel populations indicated by black and red in the FACS plots and circles and squares in

277 the graphs. For example, in the 0-13 hour samples of a 24 hour experiment, palbociclib was

278 removed at the start of sampling, while the release had been done 13 hours earlier for

279 samples that were simultaneously collected for the 13-24 hour samples. Consequently,

280 many graphs we present, have 12/13 hour time points from each set.

281

282 As the second cycle after release is less likely to be influenced by the physiological

283 challenges of induction synchrony, it is often desirable to monitor this second cycle, rather

284 than the first cycle after release [1]. We therefore extended our assessments to monitor

285 total DNA content over 48 hours after release from 24 hour treatment with 150 nM

286 palbociclib. A notable degree synchrony persisted in the second cycle with 2N cells declining

287 to constitute 46% of the population 30 hours after release (Figure 2a; Supplementary Figure

288 1). Thus, it will be possible to monitor some trends in biochemical behaviour associated

289 with cell cycle progression in the second cycle after release.

290

291 When studying events at the end of the cycle, the progression of the fastest cycling cells in

292 the population into the next cell cycle to initiate a second round of cell cycle events before

293 the events in the first cycle are completely finished in other members of the population, can

294 lead to a merged data dataset in which information from the second cycle is superimposed

295 upon that from the first to obscure finer points of the kinetics of change in the first cycle. We

296 therefore monitored the impact of palbociclib re-addition, after release from the first arrest,

297 to ask whether it would compromise the progression through the observed cycle.

298   Encouragingly, a second application of Cdk4/6 inhibitor 12 hours after release had no impact

299   upon progression through cycle under study (Figure 2b, compare the red (no re-addition)

300   and black squares (palbociclib added back at 12 h): Supplementary Figure 2a,b). Thus,

301   palbociclib re-addition can be used to insulate observations from consequences of entrance

302   into the next cycle to provide a better insight into the kinetics of cell cycle events.

303

304   **Growth conditions are key for optimal synchronisation**

305   The impact of metabolism, growth control, quiescence upon the control of commitment to

306   the cell cycle prompted us to assess the impact of context upon the efficiency of palbociclib

307   induction synchronisation of hTERT-RPE1 cells. There was a notable reduction in the

308   efficiency of synchronisation of hTERT-RPE1 cells when the density of the population seeded

309   onto plastic 6 hours prior to palbociclib addition was increased 4 fold to 1.76×10^4 cells per

310   cm^2. At this density the proportion of the population with a 2N DNA content only declined to

311   38% rather than the dip to 16% in the identical population plated at 4.4×10^3 per cm^2 (Figure

312   2c: Supplementary Figure 2c). The efficiency of both arrest and release was also

313   compromised when the identical population used in Figures 2b and 2c had been grown to

314   confluence before splitting to generate the populations that were arrested for the

315   synchronisation (declined to only 23% 2N, Figure 2d: Supplementary Figure 2d). Finally, in

316   simultaneous studies of cells from the same initial population that had been passaged twice

317   in Roswell Park Memorial Institute 1640 Medium (RPMI) before synchronisation in this

318   medium*,* fewer cells arrested cell cycle progression after 24 h in RPMI 1640 (81% vs. 95%)

319   and the proportion of 2N cells only dipped to 37% of the population in RPMI, rather than the

320   decline to 16% in DMEM (Figure 2b, e: Supplementary Figure 2a, e). Thus, culture conditions

321   alter the efficiency of synchronisation and, once initial studies indicate that a line is

322   competent for synchronisation, a variety of conditions should be assessed and care should

323   be taken to ensure that cells remain within active proliferation in the expansion in the lead

324   up to synchronisation.

325

326   **Oscillations in established cell cycle markers accompany progression through synchronised**

327   **cycles**

328   To assess the utility of the approach for biochemical assays, extracts from a palbociclib

329   synchronised culture were probed with antibodies to monitor markers whose levels

330   fluctuate as cells transit cycles synchronised by other means. Anticipated fluctuations in the

331   levels of the kinesin 5 Eg5 and phosphorylation on serine 10 of histone H3 highlight the

332   utility of this approach to monitoring biochemical changes throughout the population

333   (Figure 3).

334

335   **Synchronisation of multiple lines with palbociclib, ribociclib and abemaciclib**

336   We next asked whether palbociclib induction synchronisation would be similarly effective in

337   other lines by assessing the efficiency of arrest and subsequent release into nocodazole

338   arrest of 25 cell lines following exposure to a range of palbociclib concentrations. Although

339   far from exhaustive, these exploratory assessments identified reproducible arrest and

340   release in 5 of the lines tested. We illustrate this efficacy with THP-1, H1299, A549 (Figure

341   4a-c). The following lines did not show robust arrest/release profiles in our preliminary

342   tests: Hela, U2OS, MCF10A, HCT116, RKO.

343

344   As the different Cdk4/6 inhibitors exhibit distinct pharmacological responses [54], we

345   compared induction synchronisation profiles in different lines to gauge the spectrum of

346   responses and whether a reliance upon palbociclib alone as a means to judge competence

347   for CDK4/6 inhibition induction synchrony was a valid approach, or whether other inhibitors

348   may show even greater efficacy. A549, H1299, THP1 and hTERT-RPE1 populations were

349   exposed to a range of palbociclib, ribociclib and abembaciclib concentrations, before the

350   CDK4/6 drug containing medium was swapped for medium containing nocodazole 24 hours

351   into the arrest. These nocodazole containing cohorts were then harvested 24 hours after the

352   swap into nocodazole (Figure 4).

353

354   In keeping with the efficacy of palbociclib, these selected lines showed strong Cdk4/6

355   inhibitor induction synchrony with the two other inhibitors (Figure 4). Palbociclib and

356   ribociclib gave excellent and comparable arrest/release profiles across a broad range of

357   concentrations. In contrast, the window of competence was much narrower for abemaciclib

358   (Figure 4). When the efficiency of G1 arrest is used to identify concentrations of drug that

359   have comparable impact upon restriction point passage, the ability to exit the G1 arrest into

360   the G2 block was markedly lower when the inhibition had been imposed by abemaciclib. For

361   example, for hTERT RPE1 cells, 200 nM of abemaciclib is required to attain the same block in

362   G1 as 100 nm palbociclib, yet after release 57% of the population remained arrested with

363   2N, rather than the 15 % that persists in the palbociclib treated population (Figure 1b, Figure

364   4d). This inefficiency is a recurrent theme in all lines (Figure 4a-c). Comparisons between

365   palbociclib and ribociclib reveal subtle distinctions to suggest that, when extensive work is to

366   be performed with a specific line, there would be merit in testing both inhibitors when fine

367   tuning the dose-response profile.

368

369   The AML derived line THP-1 is an attractive line for cell cycle studies because cell retrieval

370   via pelleting avoids the challenges of harvesting from adherence to a matrix. We therefore

371   assessed both the robustness of synchrony in this line (Figure 5a, c, d, Supplementary Figure

372   3) and the behaviour of signature cell cycle markers as a population transited a synchronised

373   culture (Figure 5a,c). As noted for hTERT-RPE1 cells in Figure 3, markers oscillated with their

374   characteristic periodicities (Figure 5b).

375

Inhibitor cocktails match the efficacy of single agent synchronisation

377

378   Off-target inhibition is a concern for interpretation of phenotypes arising from chemical

379   perturbations. With the goal of minimising the off target impacts of each individual inhibitor

380   [54], we asked whether a cocktail of all three inhibitors would be as effective as single

381   agent? We used a mix of one third of the most effective concentration for each individual

382   drug to test the level of cell cycle arrest and release: 33 nM abemaciclib + 33 nM palbociclib

383   + 300 nM ribociclib.   Encouragingly, all lines gave robust arrest and efficient release (Figure

384   6).

385

γ-H2AX staining reveals minimal DNA damage in palbociclib induction synchrony

387

388   A major limitation of the widely used "double thymidine block" approach is the

389   accumulation of DNA damage during the early S phase arrest [20]. This damage likely arises

390   from collapse, and attempts to repair of the DNA replication forks that stalled because

391   nucleotide provision was compromised [19]. The damage accrued, is likely to account for the

392   abnormal anaphase profiles in the divisions after release from thymidine block [21].

393

394   We therefore monitored the accumulation of a marker of DNA double strand breaks, γ -

395   H2AX foci, to assess the level of damage during palbociclib arrest and the ensuing cycle after

396   release. As these foci form naturally during S phase, when replication forks generate double

397   strand breaks, we counterstained cells to identify cells undergoing DNA replication in the

398   hour before sampling by adding 10 µM of the nucleotide 5-ethynyl-2'-deoxyuridine (EdU)

399   one hour before processing each sample. Thus, all EdU positive cells will have been actively

400   replicating DNA in the hour before fixation. This enabled us to distinguish EdU positive cells

16

401   with γ-H2AX foci, in which we assume the foci are a consequence of DNA replication, from

402   those with no EdU staining in which we assume the foci are indicative of sites of repair of

403   DNA damage. We counted a cell as positive for γ-H2AX foci when immunofluorescence

404   staining revealed more than two foci in a nucleus. To consolidate the insight into the timing

405   of S phase from the assessment of total DNA content throughout the population (Figure 7a,

406   b), we monitored progression through S phase by quantifying the cumulative incorporation

407   of EdU into the DNA after the addition of the lower concentration of 1 µM EdU at the time

408   of release to label of all DNA synthesized after the release. S phase was largely complete by

409   16 hours after release from 150 nM palbocilib (Figure 7c).

410

411   Figure 7 d shows plots from a population of hTERT-RPE1 cells synchronised by palbociclib

412   arrest/release show the frequency of cells incorporating the EdU pulse, those showing two

413   or more γ-H2AX foci and those showing staining with both. For the plots in Figure 7e we just

414   scored cells that contained γ-H2AX foci. The portion of cells in the population with foci, yet

415   no EdU in the darker shading, while those S phase cells staining positive for EdU

416   incorporation are represented by lighter shading (Figure 7e).   These plots in Figure 7e show

417   that the vast majority of γ-H2AX positive cells are those replicating. Very few cells in the

418   population had the DNA damage marker, but no EdU staining. Furthermore, cells that have

419   only just initiated S phase may not have incorporated detectable levels of the EdU

420   nucleotide pulse, even though they will have γ-H2AX foci associated with replication forks.

421   Consequently, the assessment by scoring a positive incorporation of the EdU pulse label will

422   give a modest under estimate of S phase cells. Taking this into consideration, and the fact

423   that many cells that incorporated the EdU pulse did not have any γ -H2AX foci (Figure 7d), it

424   would appear that minimal DNA damage accompanies palbociclib induction synchronisation

425   (Figure 7d,e).

426

427   As hTERT-RPE1 cells are refractory to synchronisation with thymidine, we used H1299 to

428   directly compare the levels of DNA damage arising during palbociclib induction synchrony

429   and that accumulating at the cell cycle arrest and release following transient addition of 2

430   mM thymidine to the same starting population (Figure 8, Supplementary Figure 4). Although

431   the degree of synchrony achieved by palbociclib arrest release is not as high in H1299 as in

432   hTERT-RPE1, the results were clear. Consistent with previous reports [20], and in stark

433   contrast to the minimal levels of DNA damage with palbociclib induction synchronisation

434   (Figure 8 a-c), synchronisation by exposure to a single dose of thymidine led to γ-H2AX

435   positive scores for many cells after release from the thymidine arrest point in early S phase

436   (Figure 8 d-f). Importantly, there were foci in many cells that had not incorporated the 1 h

437   EdU pulse label at the time of fixation (the hallmark of actively replicating cells) in cells a

438   long time after the block had been released (Figure 8 e,f). These data suggest that some

439   damage that accumulated at the arrest point persisted throughout the subsequent release,

440   beyond the period of replication. Persistence of damage is consistent with previous reports

441   of chromosomal aberrations during divisions synchronised by thymidine induction synchrony

442   [21].

443

444   Although we cannot exclude forms of DNA damage that do not generate γ-H2AX foci,

445   palbociclib induction synchronisation of cell cycle progression does not appear to

446   compromise genome integrity to the same degree as thymidine based synchronisation.

447

448   **Competence to release maintained over 72 hours**

449   Removal, induction or replacement of a molecule of interest gives great insight into its

450   function. When the manipulation is done in synchronised cultures, it is imperative that the

451   manipulation does not occur in the preceding cycle, otherwise some of the phenotype

452   observed could be an indirect consequence of irrelevant damage accrued in the preceding

18

453   cycle as cells approach the block point. While impossible to avoid in selection

454   synchronisation, it remains a major challenge in many forms of induction synchronisation

455   that rely upon arrest *within* the cycle. One major appeal of halting cell cycle progression at a

456   point when one cycle is complete and the next is yet to start, is that the impact of any

457   molecular manipulation of the arrested population will generate a phenotype that is a direct

458   consequence of this manipulation upon progression through the ensuing cycle. None of the

459   consequences will be attributable to problems in completing the cycle leading up to the

460   block from which the cells are released.

461

462   We therefore compared the competence to return to cycle after Cdk4/6 inhibition for 24, 48

463   and 72 hours. Pilot experiments established that the ability to hold the arrest, or release

464   after arrest, varied between cell lines and so we selected different palbociclib

465   concentrations to rigorously quantitate the competence to release following protracted

466   arrest (Figure 9). The efficiency of synchronisation was assessed once more by using release

467   into nocodazole to trap cells in the next mitosis as a consequence of activation of the spindle

468   assembly checkpoint [58]. While the ability to maintain the G1 arrest declined to varying

469   degrees in the different lines, with hTERT-RPE1 being the most proficient at maintaining

470   arrest, all lines exited the arrest to reduce the number of 2N cells after 24 hours in

471   nocodazole (Figure 9).

472

473   **Discussion**

474   Our examination of the potential of Cdk4/6 inhibition as a novel approach to induction

475   synchrony reveals a highly effective approach to synchronisation of cell cycle progression

476   throughout a population of either adherent, or suspension, human cell lines. We believe

477   that a number of attributes make it a valuable addition to the broad portfolio of cell cycle

478   synchronisation technologies.

479

480 The release from palbociclib-imposed cell cycle arrest at the natural decision point for cells,

481 the restriction point, is not associated with changes in growth rates or rates of progression

482 through the cycle into which they are released [25,59]. Rather Cdk4/6-Cyclin D activity

483 appears to set size control [25,59]. Conceptually, this resonates with the original definition

484 of the restriction point, as a rate limiting gateway upon which multiple regulatory systems

485 converge to regulate passage through the gateway into commitment to division [60].

486

487 A second appeal of this approach lies in the apparently limited impact upon genome

488 integrity. Although the double thymidine block approach has predominated for over 50

489 years, the DNA damage that accompanies the arrest persists through the release (figure 8)

490 [20,21]. This cumulative damage limits the utility of this approach for the study in several

491 fields, including DNA replication, DNA repair and chromatin. While assessing genome

492 integrity through the acquisition of γ-H2AX foci is not an exhaustive assessment of damage,

493 our data suggest that Cdk4/6i induction synchrony is not accompanied by the damage and

494 mitotic chromosomal aberrations that arise following release from thymidine [21]. Certainly

495 extensive analyses of mitotic progression following palbociclib induction synchronisation

496 [57] revealed no sign of any of the chromosomal abnormalities that accompany thymidine

497 induction synchronisation (Jon Pines and Mark Jackman personal communication). Cdk4/6i

498 induction synchronisation therefore has the potential to open up a number of previously

499 intractable questions to study in synchronous cultures.

500

501 Perhaps the most important appeal of this approach is that cells can remain arrested outside

502 of the cell cycle in full serum over very protracted timescales while retaining the

503 competence to return to a synchronised cycle. As such, this approach will lend itself to

504 protein depletion, induction and molecular replacement while cells are out of cycle for

505 detailed interrogation of the cell cycle modulated attributes of a protein of interest at a level

506 that has been beyond reach to date. Viol and colleagues demonstrate the power of protein

507 induction at the palbociclib arrest point in their study of regulation of centriole biogenesis by

508 the kinase Nek2A [56].

509

510 While the benefits of this approach are particularly attractive, as with all approaches to cycle

511 synchronisation, disadvantages will inevitably emerge as these protocols are more widely

512 adopted. Although it seems that cell mass accumulation at the arrest point does not impact

513 upon cell cycle kinetics after release [25], studies in model organisms suggest that there is

514 likely to be adaptation to modified size control at some point after commitment [61].

515 Recent studies suggest that adaptation is unlikely to occur after the second cycle after

516 release [59]. The approach is also not effective in many lines when the level of control

517 exerted by Cdk4/6 vs Cdk2 is tipped more heavily in favour of Cdk2 control [10,41,47-

518 49,59,62-68].

519

520 When a particular line is refractory to Cdk4/6 induction synchronisation, several approaches

521 may switch the line to confer sensitivity to Cdk4/6 inhibition.   For lines in which Cdk

522 inhibition alone has little impact, reducing flux through to Cdk4/6 Cyclin D by reducing

523 serum, or inhibiting the downstream MAP kinase pathways can compromise translation to

524 reduce Cyclin D levels and tip the balance to impose cell cycle arrest by palbociclib, or Cdk2

525 inhibition [10,45,59]. The cell cycle arrest in HCT116 when Trametinib complements

526 palbociclib is a good example of this synergy [69]. The recent revelation that the Cdk4/6

527 inhibitors are targeting the inactive, rather than active Cdk4 and Cdk6 complexes, suggests

528 another option for refractory lines when resistance arises from greater reliance upon Cdk6,

529 rather than Cdk4 [41]. Cdk4/6 inhibitors impose the arrest because they sequester Cdk4

530 and Cdk4-CyclinD away from the Hsp90 chaperone system to reduce the number of

531   molecules that can form an active complex with p27 [41,53,70]. The lower affinity of Cdk6

532   for the Hsp90 chaperone complex enables it to more readily assemble into active trimers

533   that have no affinity for the inhibitors than Cdk4 does. This has prompted the suggestion

534   that the predominance of Cdk6-Cyclin D-p27 trimers depletes p27 from the pools of Cdk2 to

535   elevate Cdk2 activities and confer palbociclib resistance in cancers in which Cdk6 expression

536   is elevated [53,71,72]. Thus, cell lines that rely upon Cdk6 rather than Cdk4 to drive passage

537   through the gateway into the cycle will have reduced sensitivity to Cdk4/6 inhibitors. In such

538   lines, a Cdk6 specific PROTAC may reduce reliance upon Cdk6 to impose sensitivity to Cdk4/6

539   inhibition and so support synchronisation with the inhibitors [73]. Finally, because many cell

540   lines will bypass the requirement for Cdk4/6 by exploiting Cdk2 to drive cells into cycle,

541   partial inhibition of Cdk2 in these lines [10,74-76] should sensitize cells to Cdk4 inhibition.

542   One challenge with this approach is the accompanying risk that strong Cdk2 inhibition will

543   impact not only upon DNA replication in the preceding cycle, but upon the regulation of

544   mitotic commitment as Cdk2-Cyclin A has been tied directly to regulation of Wee1 and the

545   G2/M transition [10,77-79].

546

547   Given the variety of means by which resistance to Cdk4/6 inhibitors arises, unless the

548   genomics indicate a clear resistance to Cdk4/6 inhibition, such as loss of Rb, it is perhaps

549   easiest to empirically test whether a line will be amenable to Cdk4/6i induction synchrony.

550   One of two simple assessments will identify a line as being Cdk4/6i induction synchrony

551   compliant. The arrest/release into nocodazole that we show in Figures 1 and 4 will indicate

552   competence of most lines to synchronise by Cdk4/6i induction synchrony: a line will

553   synchronise if a population accumulates 2N DNA content one doubling time after inhibitor

554   addition, before switching to 4N DNA content a further doubling time after release into

555   nocodazole. However, this assay relies upon the strength of the spindle assembly

556   checkpoint (SAC) [58], that can be so weak in some lines that it fails to impose a long cell

557   cycle arrest with 4N DNA content [80]. The second approach is independent of SAC integrity.

558   Cells are released from palbociclib into 1μM EdU before entrance into the next cycle is

559   blocked by re-addition of palbociclib 12 hours after release. If EdU is incorporated into most

560   genomes throughout a population, that has arrested cell cycle progression with a 2N DNA

561   content in response to the second dose of palbociclib, then the line will be competent for

562   synchronisation.

563

564   Our manipulations of cell density and culture history highlight the importance of growth

565   state and culture history when customising the synchronisation protocol to gain maximum

566   synchrony in the chosen line. The plasticity of the G1 control of commitment to the cycle is

567   well documented. A cell's history alters the configuration of the re-entry switch in both

568   actively cycling cells and those returning from quiescence [45,59,81,82]. Thus, a recent

569   history of contact inhibition is likely to reduce the efficiency of synchronisation, in much the

570   same way it impacts upon the loading of replication factors at origins in hTERT-RPE1 cells

571   [83]. Consequently, care should be exercised to ensure that a population's path to

572   synchronisation has been freely dividing and as "healthy" as it can be.

573

574   While we outline the utility of palbociclib induction synchrony in the study of cell cycle

575   control and execution, pausing the cycle at the restriction point could have considerable

576   benefit in other fields, such as synchronising the formation of the primary cilium by serum

577   depletion from Cdk4/6i arrest, or in studying differentiation in systems, like haematopoesis,

578   where a pause in G1 can be critical for differentiation.

579

580   The successful quest to generate drugs to control proliferation in the clinic [51] has

581   generated tools that promise to be of great utility in cell biology and biochemistry

582   laboratories.

583

Conclusions

585 Cdk4/6 induction synchrony is a simple, reproducible, approach that will support

586 biochemical interrogation, induction, depletion and replacement of molecules in any scale of

587 cell line culture. Simple assays can determine competence of this approach to synchronise

588 cell cycle progression in a novel line. A number of approaches can be expected to convert a

589 recalcitrant line into a Cdk4/6i synchronisation compliant line.

590

Competing interests

592 We have no competing interests to declare.

593

Author Contributions

595 IH conceived the study and designed the experiments with ET who executed every

596 experiment before IH wrote the manuscript with support from ET.

597

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606

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869

870   **Figure Captions**

871   **Figure 1: Palbociclib induction synchrony of hTERT-RPE1 cells**

872   (a) hTERT-RPE1 cells were grown to 1.5×10^4 cells cm^2 in DMEM (+10% serum), trypsinised,

873   and plated at 4.4×10^3 per cm^2. 6 h later 150 nM palbociclib was added before the culture

874   medium was aspirated and replaced with DMEM + 330 nM nocodazole 24 hours later.

875   Samples were stained for PI FACS analysis at the following time points: just before

876   palbociclib addition (U - untreated), at the switch from palcociclib to nocodazole medium (P)

877   and 24h after this switch to nocodazole (P + N). The strength of the nocodazole induce

878   spindle checkpoint arrest 24 h after the nocodazole was added to an asynchronous

879   population is also shown (U + N). The bimodal peak in the upper panel (U) shows 2N DNA

880   (G1, left) and 4N (G2/M, right) DNA content of an asynchronous, untreated population. (b)

881   Cell populations were treated in the same way as panel (a) with the palbociclib

882   concentration changed to the indicated value. The frequency of 2N cells is plotted at the

883   palbociclib (grey bars) and nocodazole (blue bars) arrest points. Error bars show 1 x SD n =

884   at least 3. (c, d) Population grown as (a) with the exception of release into DMEM without

885   nocodazole and sampling every hour and cells were left for 12 hours after plating, to

886   generate the PI FACS profiles in (c) from which the plots of 2N content shown in (d) were

887   derived. The numbers next to the plots in c indicate time (hours) since release with U

888   indicating an untreated control population. The 13 – 25 h plots (red (c): open squares (d))

889   were taken simultaneously alongside the 0 - 12 h population (grey (c): filled circles (d)). n =

890   at least 3

891

892   **Figure 2: Context and perdurance for palbociclib induction synchronisation**

893   hTERT-RPE1 cells were grown as for Figure 1c with the exception that (a) sampling of 12

894   hour batches covered a 48 hour release period of the same population of cells. Samples for

895   the 0-12 (filled circles), 14-24 (open squares), 26-36 (filled circles) and 36 to 48 (open

896   squares) were taken in parallel from sub-populations to which the palbociclib had been

897   added at staggered intervals. (b) Two batches of the same population of hTERT-RPE1 cells

898   were followed, in one (open symbols) 150 nM palbociclib was re-added to the population

899   12h after the initial release from palbociclib, while the other was left to transit the

900   restriction point into the second cycle (red). For (c) the density at which cells from the same

901   population used for (b) were plated was increased 4 fold to 1.76 x 10^4 cells per cm^2, while for

902   (d) the expansion of the same starting population used in (b) and (c) lacked one cycle of

903   splitting so that the starting population that was synchronised in (d) had been grown to

904   confluence before plating 12 h before palbociclib addition. (e) Cells were grown in RPMI for

905   two passages before the entire synchronisation outlined in Figure 1c was conducted in RPMI.

906   For (b-e) samples were simultaneously taken from two batches of the same population:

907   palbociclib was added to one at the start of a 12 hour sampling period (circles), while it had

908   been added to the other 12 hours earlier (squares). For the FACS plots from which these 2N

909   DNA contents were derived see Supplementary Figures 1 and 2.

910

911   **Figure 3: Cdk4/6 induction synchrony can reveal transient cell cycle events**

912   hTERT-RPE1 cells were grown as for Figure 1c and samples taken every hour for PI FACS

913   analysis (a) to gauge the fluctuations in 2N DNA content in the population (b), while

914   sampling to monitor the indicated markers by western blot every 2 hours (c). The numbers

915   next to the plots in (a) indicate hours since release with U indicating an untreated control

916   population. Both the mitotic kinesin 5 motor protein Eg5 and phosphorylation of the serine

917   of histone H3 at position 10 peak as cells return from the 4N state to the 2N state (mitosis

918   and cell division).

919

920   **Figure 4: Spectrum of responses of four different lines to three Cdk4/6 inhibitors**

921   The indicated cell lines were all passaged in DMEM and grown as for Figure 1b with the

922   indicated drug concentrations added 6 hours after splitting. Error bars indicate 1 x SD n = at

923   least 3.

924

925   **Figure 5: Utility of THP-1 suspension cell line for cell cycle analysis**

926   (a-c) A population of THP1 cells was processed as for Figure 3 with the exception that the

927   isolation of cells for the initial seeding and shift between 100 nM palbociclib medium and

928   drug-free medium was done by mild centrifugation at 300 g for 3 minutes, rather than by

929   trypsinisation, before resuspension in RPMI at a concentration of 1×10^5 cells/ml. Sampling

930   the population for PI FACS staining (a) to monitor the 2N population (c) was accompanied by

931   isolation of cells for western blotting to detect the proteins indicated in (b). The numbers

932   next to the plots in (a) indicate hours since release with U indicating an untreated control

933   population. (d) Sampling of 12 hour batches covered a 48 hour release period of the same

934   population of cells. Samples for the 0-12 (filled circles), 14-24 (open squares), 24-36 (filled

935   circles) and 36 to 48 (open squares) were taken in parallel from sub-populations to which

936   the palbociclib had been added at staggered intervals. The FACS plots from which the data

937   in (d) are derived are in Supplementary Figure 3.

938

939   **Figure 6: Cocktails support efficient arrest and release**

940   (a-d) Populations of the indicated cell lines were processed as for Figure 1b with the

941   exception that the inhibitor was a cocktail comprising 33 nM abemaciclib + 33 nM

942   palbociclib + 300 nM ribociclib and the media switch for the THP1 was achieved by mild

943   centrifugation at 300 g for 3 minutes. Samples treated with the inhibitor cocktail are

944   indicated by C, while the untreated population by U. Error bars indicate 1 x SD n = at least 3.

945

946   **Figure 7: γ-H2AX foci associated with DNA replication in hTERT-RPE1 cells**

947   (a-c) A population of hTERT-RPE1 cells was subjected to arrest release with 150 nM

948   palbociclib treatment as described for Figure 1c and batches stained with PI to measure DNA

949   content (a,b), EdU by addition of 1 μM EdU at the time of release (c), or 10 μM EdU in 1 hour

950   pulses prior to sampling collection alongside antibodies against γ-H2AX (d, e). The numbers

951   next to the plots in (a) indicate hours since release with U indicating an untreated control

952   population. (d) Plots show the frequency of cells with staining of more than two γ-H2AX foci

953   (light fill), EdU (intermediate fill), or those cells positive for both markers (dark fill). (e) γ-

954   H2AX stained cells only: γ-H2AX alone (dark), or both γ-H2AX and EdU (light), at the

955   indicated time points after palbociclib removal. For each time point at least 200 cells were

956   counted to score each characteristic as a proportion of the total counted. The two

957   populations sampled in parallel after staggered palbociclib addition are differentiated by

958   grey and red in (a), closed circles and open squares in (b) and (c), and greys and blues in (d)

959   and (e).

960

961   **Figure 8: DNA damage at arrest and throughout release in thymidine but not palbociclib**

962   **synchronised H1299 cells**

963   A population of H1299 cells was subjected to arrest by treatment with 150 nM palbociclib (a-

964   c) or 2 mM thymidine (d-f) and processed as described for Figure 7. The plots in (a) and (d)

965   were derived from the FACs profiles shown in Supplementary Figure 4.

966

967   **Figure 9: Substantial recovery from extended palbociclib imposed cell cycle arrest**

968   The indicated lines were grown and processed as described for Figure 1b with the exception

969   that cells were maintained in the indicated concentrations of palbociclib for the listed times

970   before wash out into medium containing 330 nM nocodazole. Error bars indicate 1 x SD n =

971   at least 3.

972

973   **Supplementary Material**

974   **Supplementary Figure 1: PI FACS profiles of synchronised populations in Figure 2a**

975   The plots of PI FACS analysis of the populations shown in Figure 2a. The parallel samples

976   indicated by alternating filled circles and open squares in Figure 2a are distinguished by

977   alternating grey and red shading. Numbers indicate hours since release while U = untreated.

978

979

980   **Supplementary Figure 2: PI FACS profiles of synchronised populations in Figure 2b-e**

981   The plots of PI FACS analysis of the populations shown in the respective panels of Figure 2b-

982   e. The parallel samples indicated by filled circles and open squares in Figure 2 are

983   distinguished by the grey and red shading. Numbers indicate hours since release while U =

984   untreated.

985

986   **Supplementary Figure 3: PI FACS profiles of synchronised populations in Figure 5d**

987   The plots of PI FACS analysis of the populations shown in Figure 5d. The parallel samples

988   indicated by alternating filled circles and open squares in Figure 5d are distinguished by

989   alternating grey and red shading. Numbers indicate hours since release while U = untreated.

990

991   **Supplementary Figure 4: PI FACS profiles of synchronised populations in Figure 8**

992   The plots of PI FACS analysis of the populations shown in the respective panels of Figure 8.

993   The parallel samples indicated by filled circles and open squares in Figure 8 are distinguished

994   by the grey and red shading. Numbers indicate hours since release while U = untreated.

Trotter and Hagan Figure 4

(a) *(b)* *(c)* *(d)*

Trotter and Hagan Figure 6

(a) THP1 *(b)* A549 *(c)* H1299 *(d)* hTERT-RPE1

Legend: ■ 24 h palb ■ 24 h palb > 24 h noc