Radiation and the Mammalian Cell Cycle

A. What happens when cells are irradiated with a moderate dose (about 5 Gy)?

1] **Cell Death** - happens to many of the cells, and depends in part on the cell's "age", that is, its position in the cell cycle; this is called the **age response through the cell cycle**

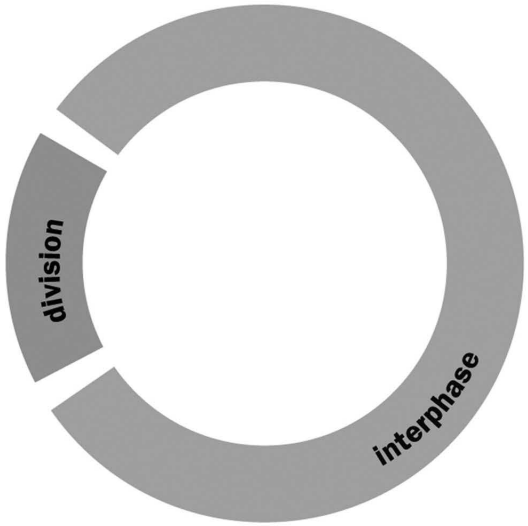
2] **Division Delay** - happens to <u>all</u> of the cells, and is a result of slowdowns and/or transient blocks, as the irradiated cells go through their cell cycle; on average, corresponds to about 1-2 hours/Gy

B. The Cell Cycle Through the Ages - a good way of putting radiation effects in their proper context

1] *The Pre-1953 Cell Cycle*:

In the (really) old days, the only cell cycle feature that could be distinguished was mitosis, identifiable microscopically by the appearance of chromosomes.

When not in mitosis, cells were said to be in the rather generic "interphase".



2] *The Cell Cycle: 1953 - 1970*:

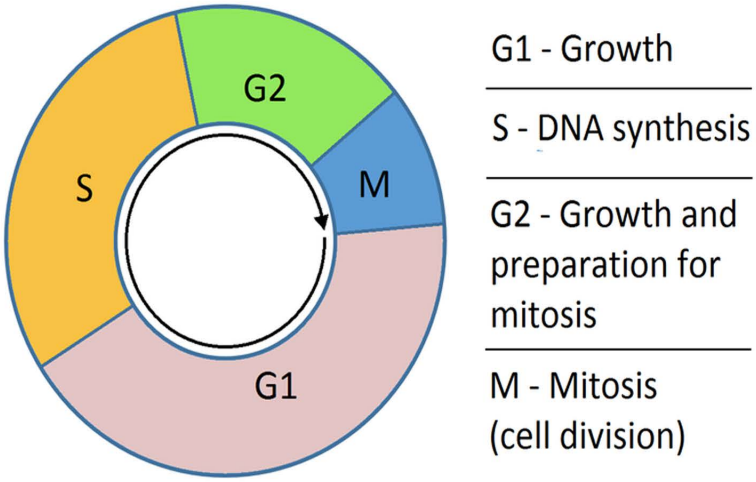
a) the identification of discrete phases within "interphase" was only made possible because a new marker was discovered that was specific for cells synthesizing DNA

1. the original marker used was ^{32}P- and ^{14}C-adenine, quickly replaced by **tritiated thymidine (^{3}H-TdR)**, a radioactively-tagged version of the nucleotide thymidine, one of the DNA bases; this marker was only incorporated into DNA when supplied to cells that were actively undergoing DNA synthesis

2. in order to detect the incorporation of the marker into individual cells, Howard and Pelc developed the technique of autoradiography (ref: Heredity *6 (suppl)*: 261-273, 1953)

(a) *autoradiography is a technique in which a liquid photographic emulsion is layered on top of a microscope slide containing fixed and stained cells, and allowed to incubate in total darkness (for upwards of several weeks!) until the low-level radioactive decays from the ^{3}H-TdR expose the emulsion*

3. thus, with two cell cycle markers available, *it was then easy to identify the other phases – G_1 and G_2 – as "gaps" falling in between the markable phases (S and M)*



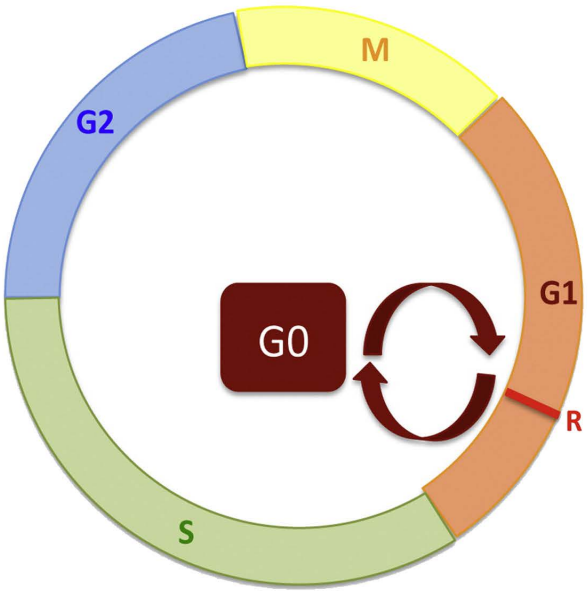
b) armed with this new knowledge that the cell cycle consisted of four phases instead of two, **researchers began to question what the actual purpose of the cell cycle was in a general sense, and in particular, what was going on during the "mysterious" phases of G_1 and G_2**

1. in the broadest sense, ***the cell cycle is the highly-regulated, uni-directional process of preparing a cell for propagation of its genetic material and for division, both being absolutely crucial during embryonic development, and later, for the growth and maintenance (and reproduction!) of the organism throughout its lifetime***

c) So is *that* the whole cell cycle story? Answer: Nope!

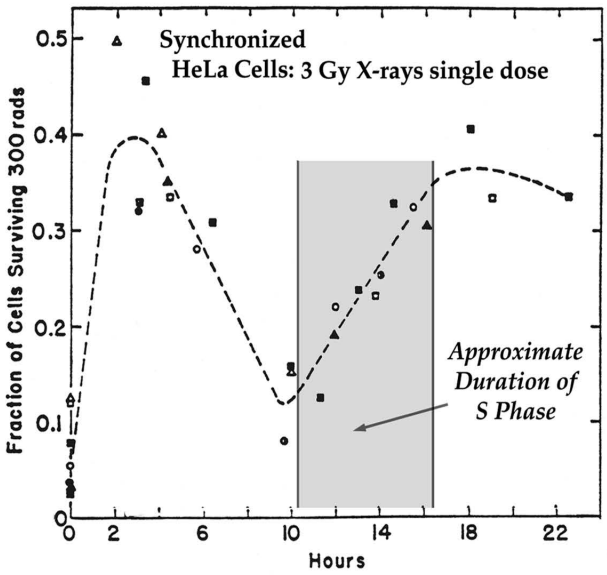
1. the idea that certain subpopulations of G1 cells were actually quiescent (temporarily out of the cell cycle, but still clonogenic and able to be **recruited** back into cycle when needed) was first introduced by Patt *et al.* in 1968--**these were called G_0 cells**

2. it was also clear that, ***while some quiescent cells were recruitable back into cycle, most were not because they were terminally differentiated and accordingly had permanently lost reproductive capacity***



Age Response Through the Cell Cycle

1. cells at different positions in the cell cycle have different sensitivities to radiation--this was first noted by Terasima and Tolmach, and subsequently studied exhaustively by Sinclair (1960's)



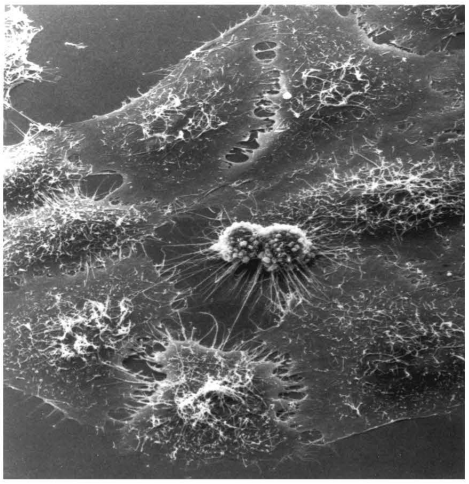
The fraction of cells surviving reproductively after receiving 300 rads of x-rays administered at different times in the division cycle, in which mitosis is taken as zero hours. Each symbol represents a separate experiment, the time scales of which have all been normalized to a minimum interdivisional time of 18 hours.

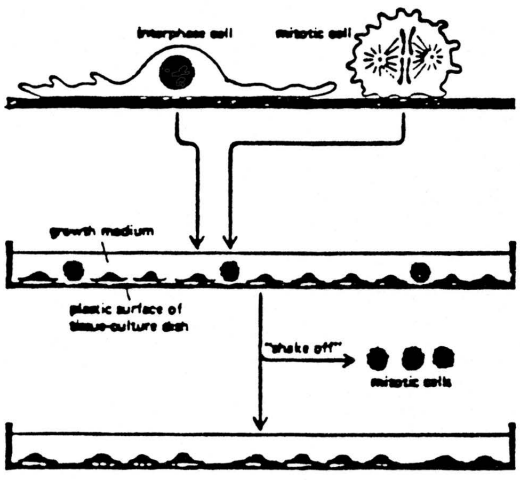
Terasima and Tolmach, Biophys J 3: 11-33, 1963

(a) in order to do these types of experiments, synchronized populations of cells are needed; how the heck did they accomplish this back in the early 1960's?

1} Answer: the **Mitotic Selection Procedure** (also developed by Terasima and Tolmach)

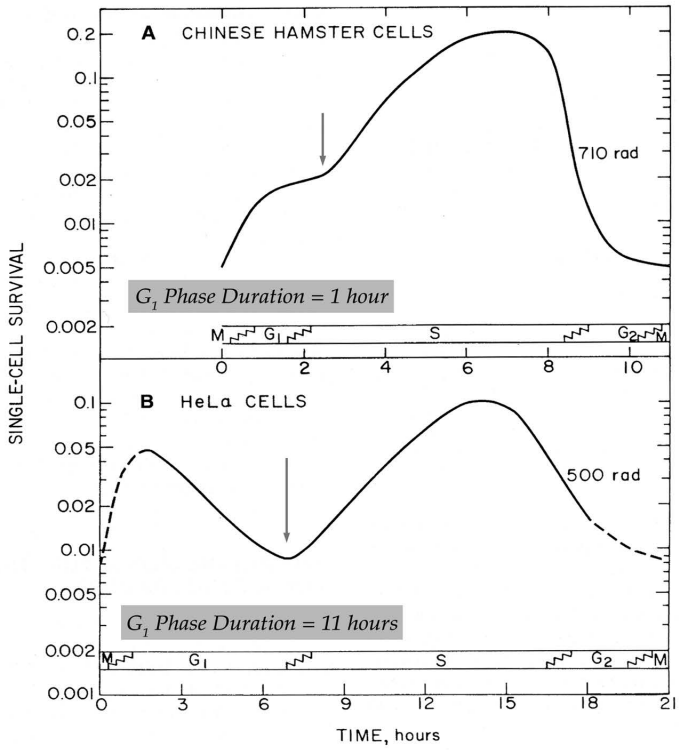
- this easy (in principle) method of synchronization takes advantage of the fact that cells in mitosis "round-up" and partially detach from the surface of a culture vessel; by shaking such a flask or petri dish, mitotic cells are dislodged into the medium and can be collected
- by repeatedly shaking flasks, collecting medium, adding new medium and shaking again at short time intervals, lots of mitotic cells can be collected with a purity of 95% or better





Mitotic cells are collected by shaking them off the dish on which they are growing. When transferred to a new dish, these collected cells continue through their cycles in synchrony.

• by leaving the cell suspension in an ice bath, cells are prevented from completing division, but as soon as cells are returned to incubator temperatures, they progress into G1, and around the rest of the cell cycle in a synchronized manner



Age–response curves for cells with short G_1 phase, represented by hamster cells (**A**), and cells with long G_1 phase, represented by HeLa cells (**B**). The time scales have been adjusted so that S phase has a comparable length on the figure for both cell lines. (From Sinclair WK: Dependence of radiosensitivity upon cell age. In *Proceedings of the Carmel Conference on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, pp 97–107. BNL Report 50203 (C-57). Upton, NY, 1969)

Radiation Age Response: General Findings

Cells in mitosis are the most sensitive to ionizing radiation, with G_2 cells not far behind.

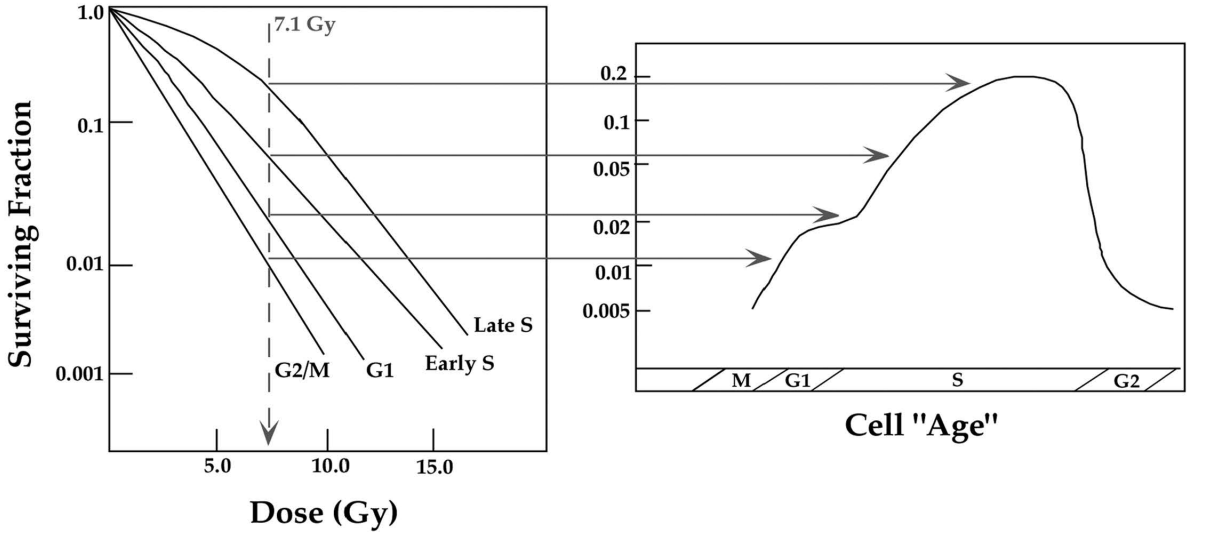
Cells are most resistant in S phase, particularly late S phase.

Cells in G_1 phase are usually of intermediate radiosensitivity, although sensitivity can vary somewhat at different points in G_1.

Also, please note the "dip" in radiosensitivity at the boundary of G_1 and S phase, that is more pronounced for human HeLa cells than rodent CHO cells. This feature is most prominent for cells with long cell cycle times (and in particular, a long G_1 phase duration)

2. How does the age response function manifest itself in terms of complete cell survival curves (as opposed to cell survival following a single dose)?

Age Response Effects in Synchronized Chinese Hamster Ovary Cells Exposed to Graded Doses of X-Rays



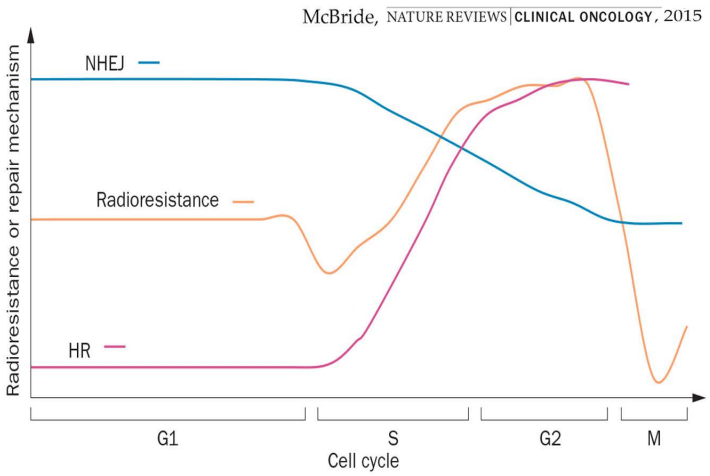
a. **G2/M cells have steep survival curves (low D_0's) and little or no survival curve shoulders**

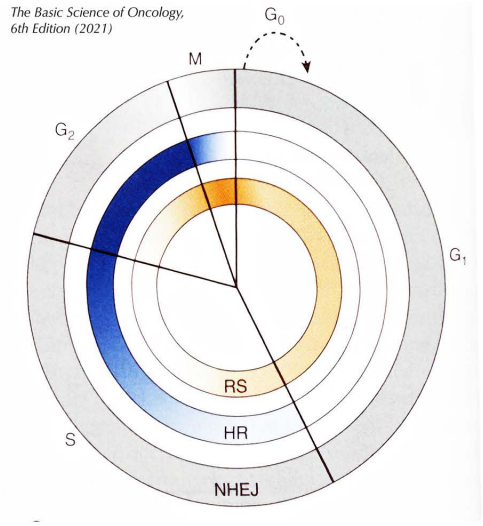
b. **late S cells have somewhat shallower survival curves (higher D_0's), but mainly, have very broad shoulders in the low dose region**

c. **G1 cells fall in between these two extremes**

3. Why is there an age response through the cell cycle?

"the DNA repair hypothesis": the newest proposed explanation for the cell cycle age response, albeit *still* not the whole story...the idea being that cells in G_1 phase repair DNA double strand breaks using non-homologous end joining, an inherently error-prone process, whereas cells in S phase repair double strand breaks using homologous recombination, an error-free process, theoretically; this might explain the greater radioresistance of S phase cells compared to G_1



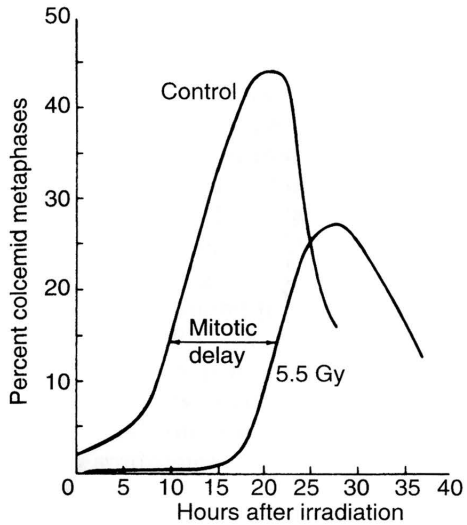


4. Is age response through the cell cycle (in tumors) important for radiotherapy?

a. this is nearly impossible to study directly, however **the phenomenon of redistribution would be a consequence of the age response** (although probably a minor effect in the grand scheme of things)

Radiation-Induced "Division Delay"

1. ***Division delay (sometimes called "mitotic delay") is an operationally-defined phenomenon characterized by a delay time in the expected appearance of mitotic cells in an exponentially growing population after irradiation, with the duration of the delay increasing with increasing dose***



The effects of radiation on the progression of cells into mitosis after the treatment. At time zero, the cells are placed in medium containing colcemid, a drug that arrests cells in mitosis, and the percentage of cells that accumulate in mitosis is plotted as a function of time. The decline in the curves at late times is a result of cells escaping the drug-induced block or dying. The mitotic delay due to a radiation dose of 5.5 Gy displaces the curves for the radiation-treated cells to the right.

From: Tannock, Hill, Bristow and Harrington, *The Basic Science of Oncology, Fourth Edition*, 2005.

a. **division delay is a consequence of radiation-induced slow-downs in transit through cell cycle phases (occurs mostly when doses are very high) and/or transient blocks at one or more discrete points upstream of mitosis (occurs even at low doses)**

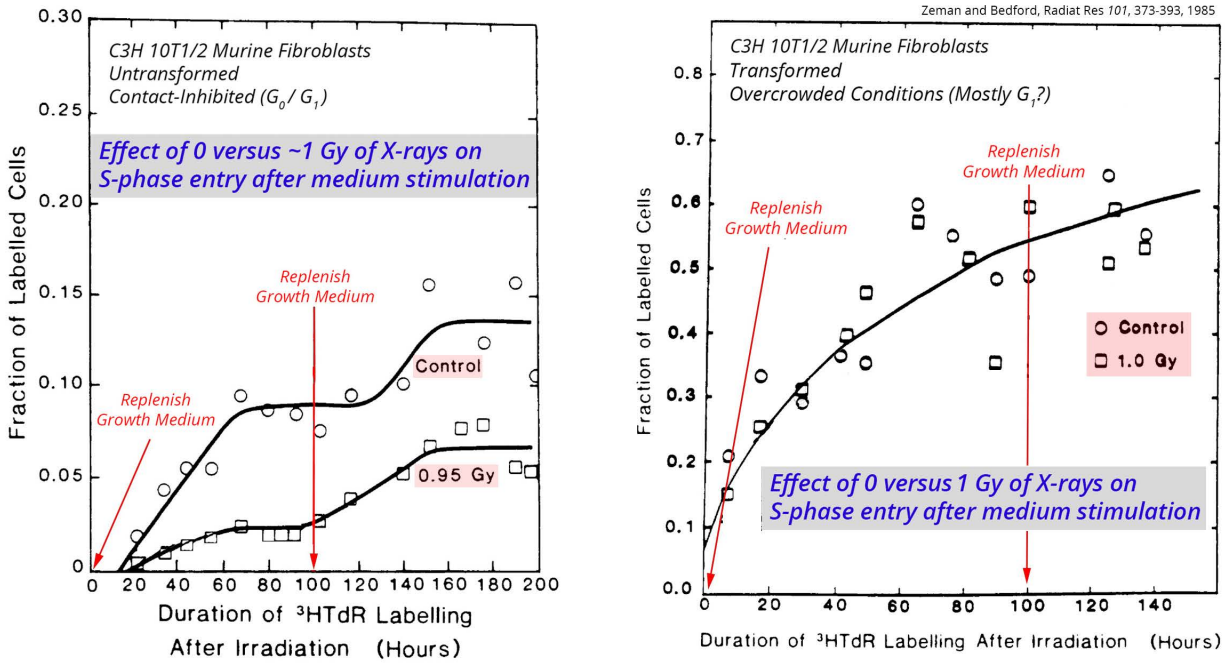
b. **G_2 Block/Delay**: historically, the major, and most studied, upstream block responsible for division delay occurs in mid-to-late G_2 phase, at a point called – back in the pre-molecular biology days – the "**X-ray transition point**" (XTP)

1) all cells, regardless of what phase they were in at the time of irradiation, and whether they will ultimately live or die, experience this block

2) *the duration of the G_2 block is cell age dependent, i.e., cells irradiated at an "age" closer to the block point (late S and early G_2) experience a longer delay than "younger" cells (G_1 or early S)...**however, on average, the duration of the G_2 block is 1-2 hours per Gy***

c. **G_1 Block/Delay**: this block is located near the border of G_1 and S phase, and also contributes to the overall amount of division delay

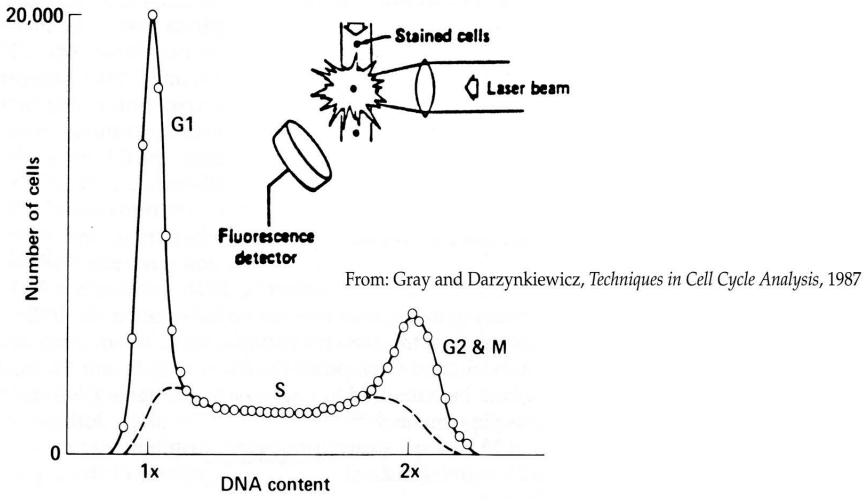
1. however, **this block is not seen in all cells, but rather is observed preferentially in slowly- or non-cycling untransformed cells (i.e., in most cases, this block is reduced or absent in tumor cells)**



An early(-ish) demonstration of a radiation-induced G_1 phase block/delay noted in untransformed mouse fibroblasts (left), but not in a transformed variant of the same cell line (right). In both cases, cells were grown to confluence, irradiated with 1 Gy of X-rays, and then "fed" with fresh growth medium. Typically, the addition of new nutrients causes a small percentage of cells to move from G_1/G_0 into S phase. ***Compared to unirradiated cells, untransformed cells receiving 1 Gy were significantly delayed in their entry into S phase, suggesting an upstream block in G_1. There is no evidence of such a delay for the transformed cells***, that readily enter S phase upon medium stimulation regardless of whether they were irradiated or not.

3] *The Cell Cycle: 1970-1985*

a) the early 1970's was a banner time for further study of the cell cycle with the development of the ***flow cytometer***, a fluorescence-based, analytical device capable of distinguishing (and ultimately, sorting) cells in different cell cycle phases on the basis of DNA content



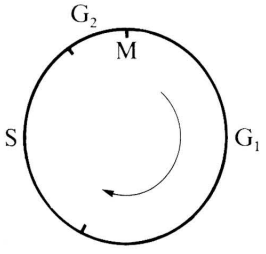
From: Gray and Darzynkiewicz, *Techniques in Cell Cycle Analysis*, 1987

DNA distribution analysis using flow cytometry involves staining cells with a fluorescent dye that binds stoichiometrically to DNA, followed by passing a stream of stained cells through a laser beam that excites the dye and emits a fluorescent signal. The signal is quantified for each cell, and a frequency histogram generated, that corresponds to the number (or fraction) of cells in each phase of the cell cycle. Phase durations can also be determined.

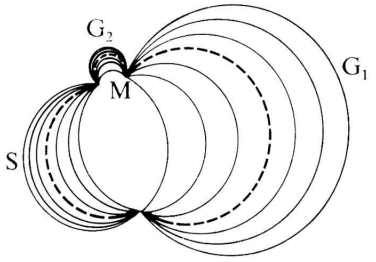
b) thanks in part to the development of the flow cytometer, it became possible to do higher resolution studies of the cell cycle, including measuring the appearance and disappearance of key proteins specific to certain cell cycle phases

1. some key findings in this regard were:

• that the cell cycle is "distributed" probabilistically, i.e., that, even for a synchronized, homogeneous population of cells, phase durations and overall cell cycle times vary around an average value



Uniform cycle



Distributed cycle

• that the variability in cycle times for different cell types was largely due to differences in the duration of G_1 phase (most of the other phases are fairly uniform in duration)

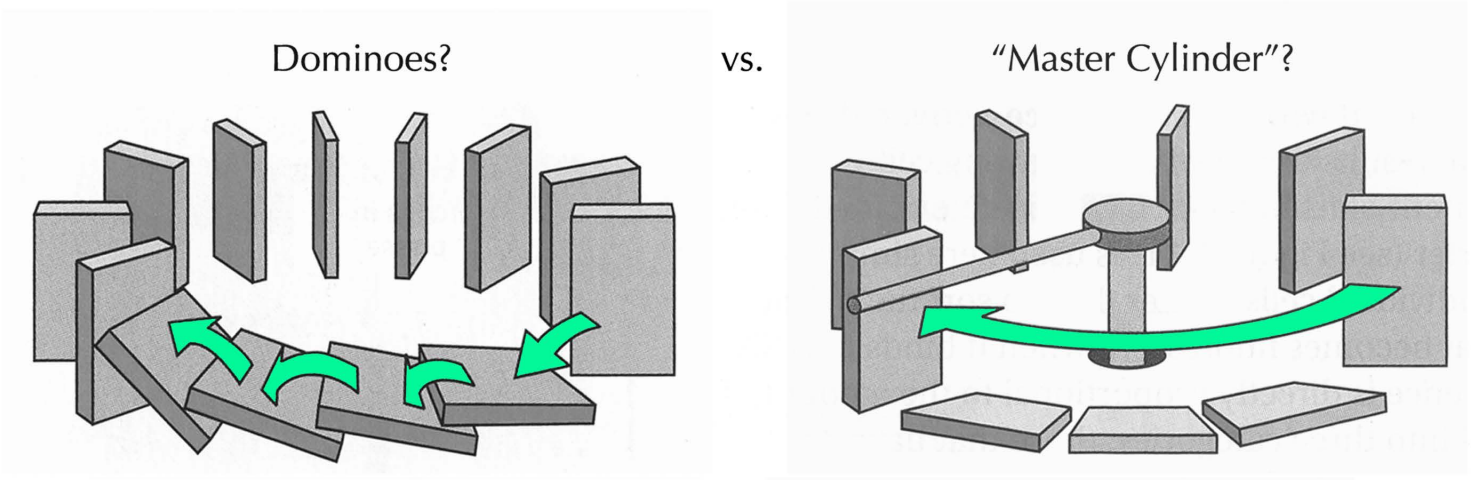
Clinical correlate: this is why attempts to synchronize tumor cells *in vivo* (in order to take advantage of redistribution of cells into radiosensitive phases) have never really worked...the synchrony is lost rapidly due to the variation in phase durations

4] *The Modern-Day Cell Cycle: driving residents crazy since 1985!*

a) by the mid-1980's, investigators began to study the molecular biology of the cell cycle, that is, what gene or genes, and their respective products actually *controlled* the movement of cells through the cycle

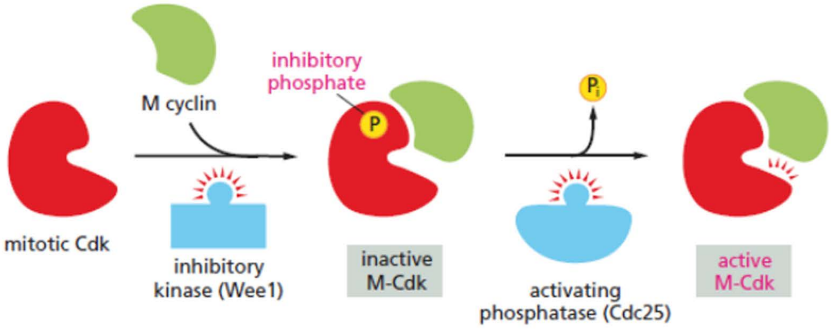
1. the first hot topic was whether movement through the cell cycle depended on a series of "events" (appearance of a protein or enzyme, etc.) that occurred in order, with one event causing the next one to occur....

...or whether a control mechanism separate from the events themselves "drove" the cell through its cycle



b) we now know that the latter model is correct, that is, that a separate set of proteins called *cyclin dependent kinases (CDK's)* drives the transition from one cell cycle phase to the next in ALL eukaryotic cells

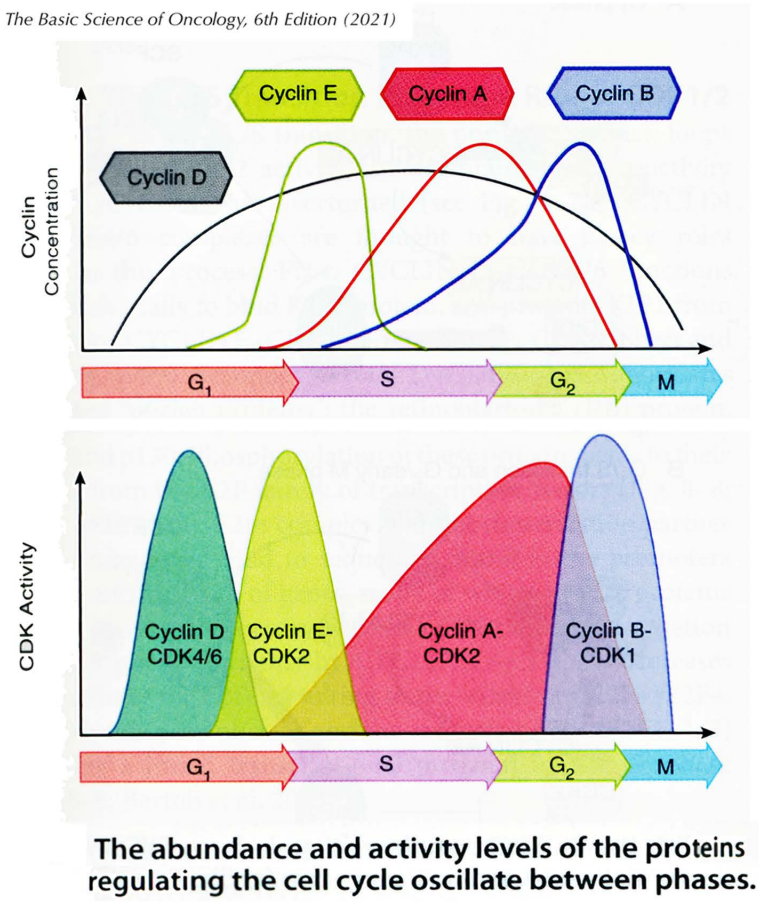
1] CDKs are activated when they bind to **cyclins**, protein partners which are differentially expressed in different phases of the cell cycle; phosphatases and kinases then act as on-off switches for the CDK-cyclin complexes



In this example, a mitotic CDK binds to its M-phase cyclin partner, but in the presence of the Wee1 kinase, the complex remains inactive.

However, once the Cdc25 phosphatase removes the phosphate group from the CDK, then the complex becomes active, and drives the cell from G2 phase into mitosis.

CYCLINS & CDK'S IN CELL CYCLE REGULATION

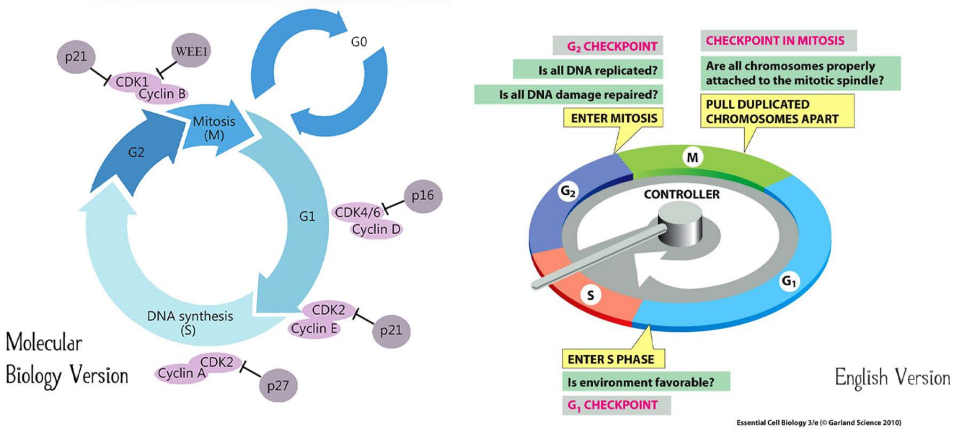


The abundance and activity levels of the proteins regulating the cell cycle oscillate between phases.

c. Question: **Why have such a complicated, convoluted, control process for the cell cycle (especially since, if one little thing goes wrong with one component of the system, the whole cell cycle becomes de-regulated)?**

1. Answer: **First, to guarantee that the cell cycle is an irreversible, one-way process, and second, to provide the cell with a quick response, "checkpoint" system that can start or stop movement through the cell cycle in response to external stimuli (e.g., DNA damage that requires repair, growth factor stimulation or lack thereof, etc.)**

The (somewhat) simplified model of cell cycle checkpoint control



C. Cell Cycle Checkpoint Control and Ionizing Radiation

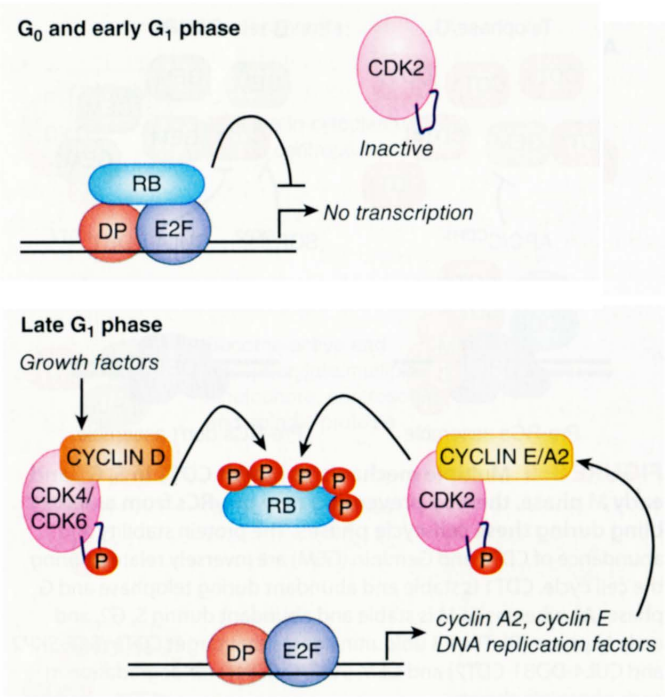
1] ***it is now clear that the radiation-induced G_1 and G_2 delays characterized decades earlier are manifestations of cell cycle checkpoint control, and in particular, checkpoints responsive to the presence of DNA damage***

2] the G_1 – S phase checkpoint: a closer look

a) there are several key players of interest to the radiation oncology community that participate in the G_1 phase checkpoint: ATM, p53 and Rb (as well as cyclins D and E, and CDK's 2, 4 and 6)

1. defects in one or more of these cell cycle regulators can lead to loss of checkpoint control and the resulting inappropriate proliferation of cells that harbor residual DNA damage – NOT a good thing generally speaking

pRB is the final link in a chain of proteins that regulate the transition from G_1 to S phase...



1. A cell in G_1 phase can't move into S phase until vital genes are transcribed, but this can't happen because RB is bound to E2F, a transcription factor.

2. When the cell receives the appropriate signals that it is ready and able to progress into S phase, the cyclin-dependent kinase CDK4 is activated through binding with its partner cyclin, Cyclin D.

This allows CDK4 to bind to RB.

3. CDK4 phosphorylates RB, causing it to undergo a conformational change. (This process is further facilitated by CDK2 and its partner, Cyclin E.)

4. The conformational change in RB causes it to "disconnect" from E2F, freeing it up to activate the genes necessary for movement into S phase.

But what happens if radiation-induced DNA damage is present?

1. ATM is activated by residual DNA damage, and in turn activates Chk2.

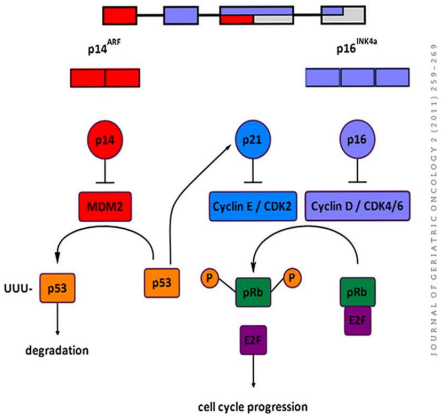
2. Chk2 inhibits (indirectly) the activity of the G_1-related CDK's, which has the immediate effect of blocking cells from moving into S phase, because pRb does not get phosphorylated.

3. Chk2 also activates p53, which in turn activates p21. p21 goes on to inactivate the CDK2-cyclin E complex, which likewise prevents pRb from being phosphorylated. This action sustains the G_1 block long enough to allow any DNA damage to be repaired prior to allowing the cell to enter S phase.

p16 (a member of the INK4 family) also plays a role in helping to maintain the G1 block by preventing the association between Cyclin D and CDK 4/6.

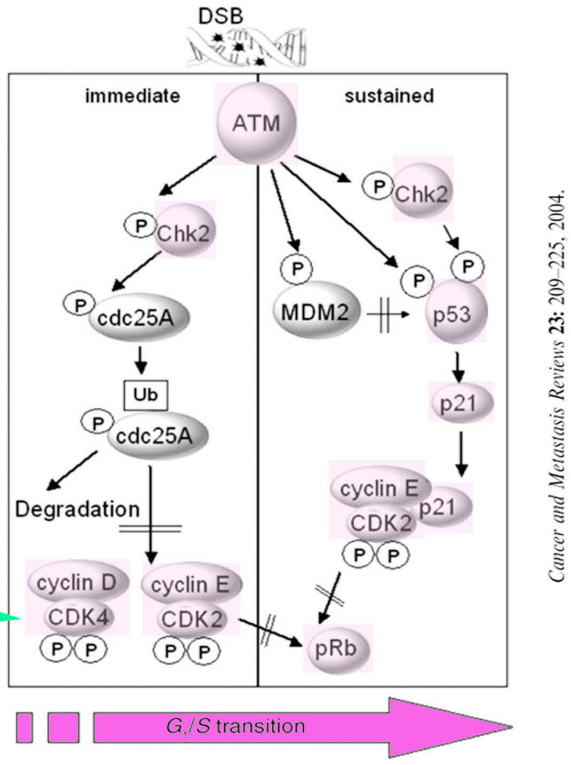
p16 does this independently of ATM and p53 though, so provides a backup system to maintain the block in case one or both of these key proteins is defective or lost.

It's only temporary though before cells start to leak into S phase. 😢



JOURNAL OF GERIATRIC ONCOLOGY 2 (2011) 259–269

Tumor suppressor protein **p16** acts here by preventing the association between Cyclin D and CDK4 (and 6)



Cancer and Metastasis Reviews **23:** 209–225, 2004.

How to "derange" the G_1 checkpoint system...

tumor suppressor genes

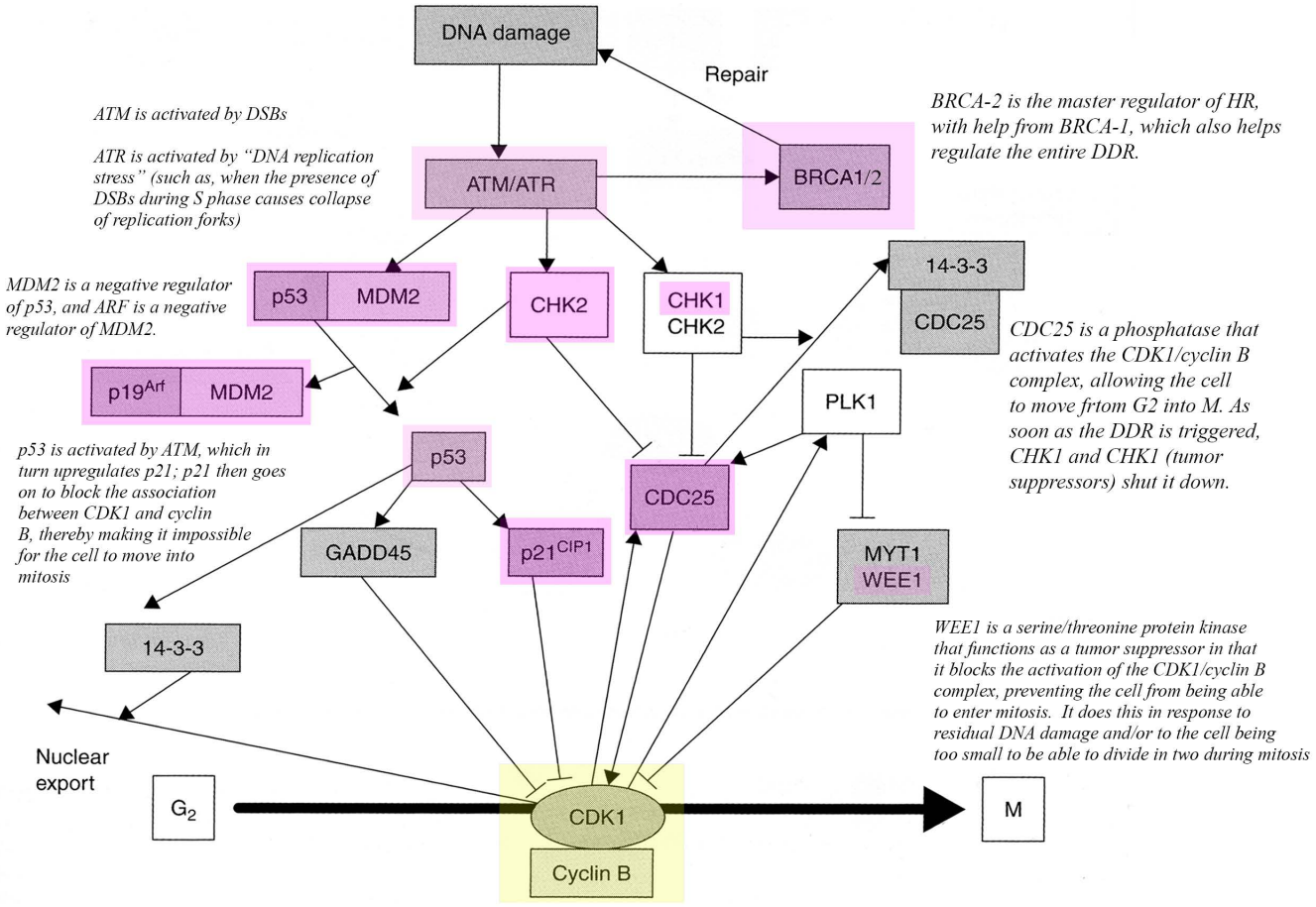
- ATM mutated or missing: cells lose the G_1 checkpoint and continue into S phase with residual DNA damage; this is often fatal (apoptosis), but if not, can predispose the cell to becoming transformed
- p53 mutated or missing: ditto; often includes loss of apoptotic pathways as well
- pRb mutated or missing: cancer proneness due to loss of G_1 checkpoint

oncogenes

- excess of G_1 cyclins and/or CDK's (especially cyclin D and/or CDK4): cells continue to proliferate inappropriately, even in the presence of "stop growing" signals
- too much p16 (or p21): would normally lead to senescence (i.e, permanent G1 arrest), however if pRB is already mutated or missing, these would have no effect...although they *would keep trying* → *explains why p16 is chronically elevated in HPV+ head & neck cancer*
- too much MDM2: would block p53 from functioning by degrading it too quickly, such that there would then be no p21, and the G1 checkpoint would become deranged
- too little p14 (ARF): inhibits MDM2 by sequestering it in the nucleus so that it can't degrade p53; loss of ARF would therefore result in too much MDM2 and too little p53 function (as above)

3] the G_2 – M phase checkpoint: a closer look

(The proteins highlighted in pink seem to be board exam favorites.)



Pelengaris and Kahn, *The Molecular Biology of Cancer*, 2006

Similar story, but somewhat different "players".

In response to residual DNA damage in G_2 cells that are waiting for the OK to enter mitosis, both ATM, ATR, CHK1, CHK2, p53, p21 and GADD45 are involved.

The ultimate effect is to prevent the association between Cyclin B and CDK1, which triggers the progression from G_2 into M.

Clinical Correlates:

1. there is considerable interest in inhibiting cell cycle checkpoint-related proteins in an effort to reign in excessive tumor cell proliferation and/or promote tumor cell death

a) *most of this work is ongoing in breast cancer, where it is already known that cyclins (especially cyclin D) and CDK's are overexpressed, and for many, that p53 and Rb are already mutated or lost*

Phase III studies of CDK 4/6 inhibitors in breast cancer — Current Oncology Reports (2019) 21: 25

Study	CDK 4/6 inhibitor	Study population	Line of therapy	Sample size	Median PFS (months) vs. placebo
Trials in combination with NSAIs					
PALOMA-2	Palbociclib	Postmenopausal women with HR+/HER2− ABC and no prior systemic treatment for ABC; (neo)adjuvant ET permitted if disease-free interval > 12 months from therapy completion	1st line	666	24.8 vs. 14.5 (HR 0.58; $P < .001$)
MONALEESA-2	Ribociclib		1st line	668	25.3 vs. 16.0 (HR 0.57; $P < .001$)
MONARCH 3	Abemaciclib		1st line	493	NR vs. 14.7 (HR 0.54; $P < .001$)
Trials in combination with fulvestrant					
MONALEESA-3	Ribociclib	Postmenopausal women and men with HR+/HER2− ABC, 0–1 line of ET for ABC[b]	1st and 2nd line	726	20.5 vs. 12.8 (HR 0.60; $P < .001$)
MONARCH 2	Abemaciclib	Women with HR+/HER2− ABC that had progressed during prior ET, any menopausal status, ≤ 1 ET, no prior CT for advanced disease[c]	2nd line	669	16.4 vs. 9.3 (HR 0.55; $P < .001$)
PALOMA-3	Palbociclib	Women with HR+/HER2− ABC that relapsed or progressed during ET, any menopausal status, ≤ 1 line of CT for advanced disease[a]	2nd line and plus	521	9.5 vs. 4.6 (HR 0.46; $P < .001$)
Trials in combination with tamoxifen or NSAI + Goserelin					
MONALEESA-7	Ribociclib	Pre/perimenopausal women with HR+/HER2− ABC, no prior ET for advanced disease, ≤ 1 line of CT for advanced disease	1st line	672	23.8 vs. 13.0 (HR 0.55; $P < .001$)

Palbociclib dose was 125 mg daily orally and ribociclib dose was 600 mg daily orally on a 3-week on, 1-week off schedule in all studies. Abemaciclib final dose was 150 mg orally twice a day continuously

NSAI non-steroidal aromatase inhibitor, *ABC* advanced breast cancer, *ET* endocrine therapy, *CT* chemotherapy, *NR* not reached, *PFS* progression-free survival, *HR* hazard ratio

b) *another experimental agent of interest, AZD1775, inhibits WEE1, causing G_2 checkpoint escape and therefore, inappropriate entry into mitosis with residual DNA damage present or with a too-small cell...both of which would cause cell death during mitosis* (currently in Phase 1/2 clinical trials in combination with other cancer therapies for patients with refractory or recurrent solid tumors)

AZD1775 now has its own drug name: **Adavosertib**

2. what still isn't clear though is how best to sequence cell cycle inhibitors with radiation and/or chemotherapy...

C. Becherini et al. — Cancer Treatment Reviews 119 (2023) 102586

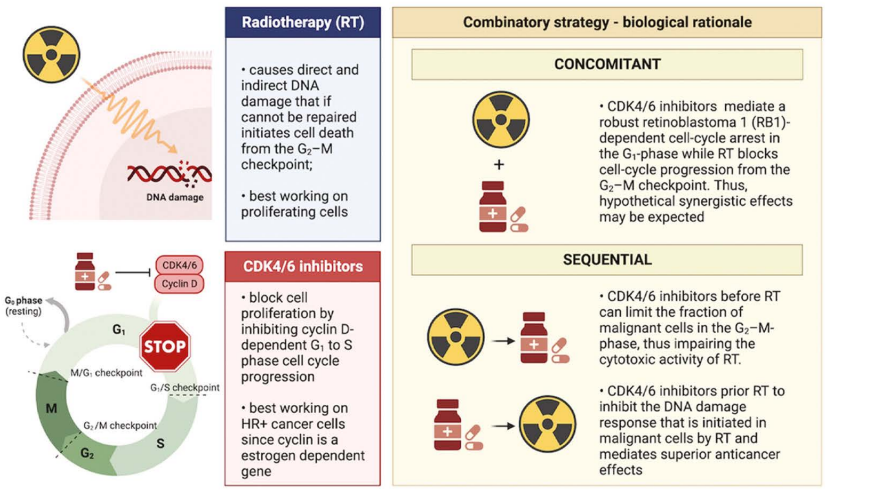
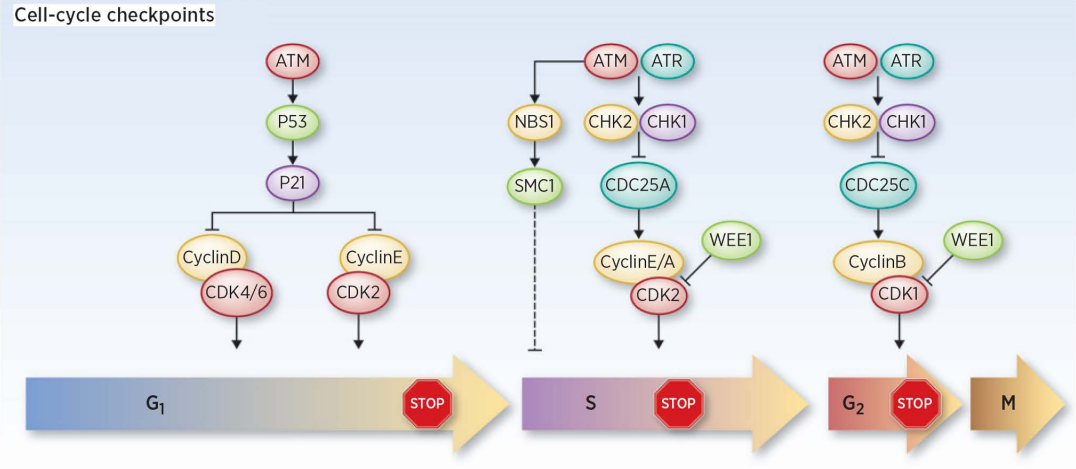


Fig. 3. Biological rationale of CDK4/6 inhibitors and irradiation combinatory strategy.

APPENDIX MATERIALS



Drugs that target cell cycle regulatory factors as possible therapeutics for cancer

Molecular target	Drugs	Clinical use
Forcing cell cycle exit		
CDK4/6	Palbociclib	Approved for ER$^+$ and HER2$^-$ metastatic breast cancer; clinical trials for multiple solid tumours
	Ribociclib	Approved for ER$^+$ and HER2$^-$ metastatic breast cancer; clinical trials for multiple solid tumours
	Abemaciclib	Approved for ER$^+$ and HER2$^-$ metastatic breast cancer; clinical trials for multiple solid tumours
CDK7	ICEC0942 (CT7001)	Phase I/II, ER$^+$ breast cancer, AML
Forcing cell cycle progression		
WEE1	Adavosertib (AZD1775)	Phase II, relapsed SCLC, ovarian cancer, NSCLC, AML, gastric adenocarcinoma and various advanced solid tumours
Impairing replication stress tolerance		
ATR	VX-970	Phase II, recurrent ovarian, primary peritoneal or fallopian tube cancer and metastatic urothelial carcinoma
CHK1	LY2606368	Phase II, SCLC, *BRCA1*/*BRCA2*-mutated breast or ovarian cancer, TNBC, HGSOC, metastatic CRPC and advanced solid tumours with HRR defects or genetic alterations indicative of replication stress.
		Phase I, acute leukaemia, solid tumours or lymphoma
	MK-8776	Phase II, relapsed AML (with or without cytarabine)
		Phase I, advanced solid tumours
Inducing catastrophic genome instability		
Mitotic spindle	Taxanes (paclitaxel, docetaxel and nanoparticle albumin-bound paclitaxel)	Approved for use in a wide range of cancers, including ovarian cancer, breast cancer, lung cancer, bladder cancer, prostate cancer, melanoma and oesophageal cancer.
	Vinca alkaloids (vinblastine and vincristine)	Approved for use in a range of cancers, including ALL, AML, HL, neuroblastoma and NSCLC
SAC	MPS inhibitors (BAY 1161909 and BAY 1217389)	Preclinical studies in neuroblastoma, medulloblastoma and breast cancer (in combination with taxanes). Recently entered phase I clinical trials
	Aurora B inhibitors (various, including AZD1152 and AT9283)	Phase II in AML, multiple myeloma, SCLC and prostate cancer. Phase I in various solid tumours

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; ATR, ataxia telangiectasia and Rad3-related protein; CDK, cyclin-dependent kinase; CRPC, castration-resistant prostate cancer; ER, oestrogen receptor; HGSOC, high-grade serous ovarian cancer; HL, Hodgkin lymphoma; HRR, homologous recombination repair; NSCLC, non-small-cell lung cancer; SAC, spindle assembly checkpoint; SCLC, small-cell lung cancer; TNBC, triple-negative breast cancer.

Nature Reviews Molecular Cell Biology **23**, 74–88 (2022)