A Continuum Model of Cell Fate in the Response of Human Cells to Ionizing Radiation

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Accessibility
A continuum model of cell fate in the response of human cells to ionizing radiation

A dissertation presented
by
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to
The Department of Systems Biology
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A continuum model of cell fate in the response of human cells to ionizing radiation

Abstract

In response to ionizing radiation induced DNA damage, human cells can either recover and resume proliferation or activate anti-proliferative programs such as cell death and cellular senescence, a state characterized by the long-term enforcement of cell cycle arrest and the loss of recovery potential. Knowledge in this field has been primarily constructed on the basis of experimental approaches that rely on averaging or static snapshots of cell populations. While invaluable insights have been gained regarding the mechanistic details of the molecular circuitry linking DNA damage sensing and downstream cellular programs, such approaches are limited in their ability to account for heterogeneity in the long-term phenotypic outcomes of DNA damage, particularly in the temporal dimension of this cell fate decision-making process. Here, we used live cell imaging to quantify the number and timing of division events that individual human cells go through in the course of one week after acute exposure to ionizing radiation. Such single cell division profiles unmasked heterogeneity both in the long-term maintenance of cell cycle arrest in senescing cells, and in the timescale of cell cycle re-entry of recovering cells.

We first present our findings on the molecular mechanisms underlying escape from cell cycle arrest in the presence of unrepaired DNA damage. Using fluorescent reporters for p53 and p21, which trigger cell cycle arrest upon DNA damage, we identified two levels at which quantitative variation in these proteins contributes to escape from cell cycle arrest: (i) low historical averages of p53 and p21 prime cells to eventually escape the arrested state; and (ii) a transient decrease in p53 levels precedes the
onset of cell cycle re-entry. Using mathematical modeling, we show that fluctuations in p53 can be amplified into a sharp switch between p21 and CDK2, and consequent cell cycle re-entry. Taken together, our work revealed that p53 and p21 dynamically contribute to the active maintenance of the arrested state, the homeostasis of which can be broken through local amplification of noisy DNA damage signaling.

We next discuss our findings on the consequences of heterogeneity in cell cycle arrest duration in the context of combination cancer therapy. We focused on the interaction between acute exposure to ionizing radiation and transient treatment with the Eg5 kinesin inhibitor STLC, which selectively targets mitotic cells. DNA damage antagonized STLC treatment through establishment of cell cycle arrest, leading to a non-monotonic dose response curve in which an intermediate dose of damage optimized cell viability. Optimal damage dose shifted as a function of STLC treatment duration. We aim to understand the extent to which input-output relationships in DNA damage signaling can modulate arrest-exit time distributions and shape the response of human cells to dynamic therapeutic strategies.

Our investigations on the long-term fate of human cells after exposure to ionizing radiation revealed a phenotypic continuum in which the discreteness of arrested and cycling states is blurred out when taking into consideration whole histories of individual cell behavior. A quantitative account of DNA damage signaling will provide a framework to understand the way human cells regulate specific properties of this cell fate continuum, including arrest duration and recovery potential, and will guide future interventions aimed at optimizing DNA damage-based cancer therapies.
Contents

1 Introduction 1
  1.1 Self-replication as an information processing problem 1
  1.2 Regulatory logic of mammalian cell cycle control: irreversible transitions, triggers and checkpoints 3
  1.3 Processing the DNA damage signal 6
  1.4 Molecular and phenotypic cell-to-cell variability: challenges and opportunities for cells and cell biologists 9
  1.5 The long-term phenotypic consequences of DNA damage 14
  1.6 A roadmap for this thesis 15

2 Noise driven escape from cell cycle arrest 17
  2.1 Individual cells exhibit heterogeneity in the long-term maintenance of cell cycle arrest 19
  2.2 Cell-to-cell variability in DNA damage signaling contributes to escape from arrest 22
  2.3 Escape from arrest is characterized by a sharp switch in p21 levels and CDK2 activity 25
  2.4 Fluctuations in p53 are amplified into escape from arrest 27
  2.5 Materials and Methods 32
  2.6 Acknowledgements 40
  2.7 Author contributions 40

3 Antagonism between anti-cancer treatments leads to non-monotonicity in the cellular response to DNA damage 41
List of Figures

1.1 Self-replication can be understood as an information processing problem. .................................................. 3
1.2 Regulatory logic within the core cell cycle control circuitry underlies irreversible cell cycle transitions. .......................... 5
1.3 p53 signaling links sensors and effectors in the response of human cells to DNA damage. ..................................... 7
1.4 Insights gained from quantifying p53 protein dynamics in individual cells. .................................................. 12

2.1 Diverse cellular phenotypes can result from exposure of human cells to ionizing radiation. ..................................... 18
2.2 DNA damage leads to heterogeneous division profiles over long timescales .................................................. 20
2.3 A subpopulation of cells escape G1 arrest over long timescales ................................................................. 21
2.4 Cells escape G1 arrest in the presence of DNA damage ................................................................. 23
2.5 Fluorescent tagging allows quantification of p53 and p21 dynamics in live individual cells .............................. 24
2.6 Cell-to-cell variation in DNA damage signaling contributes to heterogeneity in arrest maintenance .............................. 26
2.7 Escape from cell cycle arrest is characterized by a sharp switch in the balance between p21 and CDK2 activity .............................. 28
2.8 Double-negative feedback between p21 and CDK2 can act as a non-linear amplifier of upstream p53 fluctuations ........................................ 29
2.9 Noise in p53 is amplified into escape from cell cycle arrest ................................................................. 31
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10</td>
<td>Escape from arrest is associated with chromosome missegregation and re-enforcement of DNA damage signaling.</td>
<td>33</td>
</tr>
<tr>
<td>3.1</td>
<td>Ionizing radiation diversifies cellular states in isogenic cell populations</td>
<td>42</td>
</tr>
<tr>
<td>3.2</td>
<td>Both DNA damage and the Eg5 kinesin inhibitor STLC limit cell proliferation</td>
<td>44</td>
</tr>
<tr>
<td>3.3</td>
<td>Simulations suggest re-shaping of DNA damage dose response in the context of combined treatment with STLC.</td>
<td>46</td>
</tr>
<tr>
<td>3.4</td>
<td>Optimal dose of damage changes with STLC treatment schedule.</td>
<td>47</td>
</tr>
<tr>
<td>3.5</td>
<td>Strong perturbation of p53 signaling abrogates non-monotonic interaction between DNA damage and STLC</td>
<td>48</td>
</tr>
<tr>
<td>3.6</td>
<td>Levels of p21 induction correlate with cell cycle arrest duration after DNA damage</td>
<td>51</td>
</tr>
<tr>
<td>4.1</td>
<td>Month-long timecourse suggests occurrence of escape events beyond one week after DNA damage</td>
<td>59</td>
</tr>
<tr>
<td>A.1</td>
<td>p53Cinema tracking strategy at a glance.</td>
<td>70</td>
</tr>
<tr>
<td>A.2</td>
<td>p53Cinema allows tracking of individual cell lineages across multiple generations.</td>
<td>73</td>
</tr>
<tr>
<td>A.3</td>
<td>Single cell lineages reveal heritability of G1 duration.</td>
<td>74</td>
</tr>
</tbody>
</table>
List of Tables

2.1 Parameters used in computational model. . . . . . . . . . . . . . . . . . . . . 39
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I am fascinated by the question of how reproducible the trajectories of dynamical
systems are. If we were to rewind history, would such systems reach the exact same states, or is the fate of such systems contingent on unpredictable historical events? As I finish this work, it is hard for me to think of necessarily sufficient conditions that led me to share this with you. It rather seems as an accident, contingent on a series of decisions made with a great deal of uncertainty of their long-term outcome. I don’t even think of myself as deserving your attention in reading this document by virtue of necessity. Just lucky. It is my hope that you find interest in this lucky accident.

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José Reyes

June, 2017
1.1 **Self-replication as an information processing problem**

Cells have the remarkable capacity of generating two nearly identical copies of themselves through a set of biochemical processes that collectively constitute the cell cycle. Self-replication is a fundamental property of all living systems and it is the basis for the generation of all the cells that constitute a multicellular organism. Regulated cell proliferation is integral for the maintenance of adult tissues, and its deregulation is a unifying property of all forms of cancer.

Our conceptualization of the eukaryotic cell cycle is organized around phases of DNA replication and segregation (known as S- and M- phases, respectively), which are separated by phases of growth and cell cycle control (known as G1 and G2 phases) (Fig. 1).
Through accurate genome replication and segregation, daughter cells inherit a full copy of the genetic information that is necessary for the continuous production and maintenance of other constituents of the cell. Errors in these processes can have strong deleterious consequences at both the cellular and organismal levels [1–3], and also constitute a substrate for phenotypic variation, selection and evolution. In the context of long-lived multicellular organisms, such errors are recognized as prevailing drivers of tumorigenic transformation [4, 5].

Several properties of the cell cycle confer reliability to the inheritance of genetic information. First, genome replication and segregation are carried out by macromolecular machines with exquisite precision: measured error rates of replicative polymerases range from $10^{-6}$ to $10^{-8}$ per base [6] and chromosome mis-segregation rates are estimated to be in the order of $10^{-2}$ per cell division [2] (Fig. 1.1). Second, the regulatory logic underlying irreversible transitions during the cell cycle confers unidirectionality to cell cycle progression; in particular, commitment to replicate DNA ensures that genome replication is started and completed only once per cycle (Fig. 1.1, discussed in detail in Section 1.2). Finally, cells have the ability to trigger and prevent these irreversible transitions through the integration of information about their internal state and environment; of particular interest for this work are surveillance mechanisms devoted to detect and repair DNA damage, and to halt cell cycle progression and thereby limit the expansion of damaged cells (Fig. 1.1, discussed in detail in Section 1.3).

In this framework, self-replication can be understood as an information processing problem: integration of dynamic information about the cell and its environment drives unidirectional progression through a series of cellular states, which collectively allow the accurate replication and segregation of genetic information. A major goal in the field of signal processing during the cell cycle is to understand how distinct and
Figure 1.1: Self-replication can be understood as an information processing problem. Proliferative and anti-proliferative signals are integrated to bring about sharp and irreversible cell cycle transitions. Unidirectional progression through distinct phases of the cell cycle allows faithful replication and transmission of genetic information from one cell generation to the next.

often conflicting signals are integrated to regulate cell cycle transitions. A quantitative account of signaling is pivotal to understand the limits of information transfer within cells and the way changes in signal-response relationships drive pathological conditions.

1.2 REGULATORY LOGIC OF MAMMALIAN CELL CYCLE CONTROL: IRREVERSIBLE TRANSITIONS, TRIGGERS AND CHECKPOINTS

Over 40 years of research have resulted in the detailed characterization of the molecular circuitry that controls cell cycle progression and the regulatory logic underlying irreversible cell cycle transitions [7–9]. Here I summarize our current understanding of the sequence of molecular events that commit cells to genome replication, also known as G1-S transition. Although the specific molecular players differ, similar principles govern transition into mitosis [10].
At its core, cell cycle control can be understood as a set of mechanisms that converge on the modulation of Cyclin-dependent kinase (CDK) activity. CDKs associate with specific types of Cyclins in particular phases of the cell cycle and phosphorylate multiple targets to either enhance or inhibit their levels and activity, leading to global changes in the cellular state. In the case of the G1-S transition, CyclinD-CDK4/6 complexes phosphorylate the retinoblastoma (Rb) tumor suppressor protein. This modification leads to the dissociation of Rb from the transcription factor E2F and subsequent induction of downstream target genes, including E2F itself and CyclinE. CyclinE-CDK2 complexes reinforce the initial signal through Rb hyperphosphorylation and drive the start of DNA replication.

CDK activity is further modulated by a conserved family of CDK inhibitor proteins, which include p21 and p27. At the G1-S transition, CDK inhibitors sequester G1 Cyclin-CDK complexes and directly inhibit their interaction with cellular substrates. The CyclinE-CDK2 complex in turn phosphorylates p21 and p27 and targets these proteins for degradation by the SCF E3 ubiquitin ligase. Reciprocal regulation between Cyclin-CDK activity and CDK inhibitors forms a double negative feedback loop that, similarly to the E2F-dependent transcriptional arm described above, can reinforce initial CDK activation and drive a sharp transition into S-phase.

Transcriptional and post-translational positive feedback control at the level of CDK activity underlies the irreversibility and sharpness of the G1-S transition and ensures unidirectionality of cell cycle progression. This regulatory logic is conserved throughout evolution, from yeast to mammals, and similar circuit architectures are instantiated in other processes involving sharp and irreversible transitions,
Figure 1.2: Regulatory logic within the core cell cycle control circuitry underlies irreversible cell cycle transitions. CDK inhibitors such as p21 form a double negative feedback loop with CyclinE-CDK2. This circuit architecture can function as a toggle switch. E2F-dependent transcriptional positive feedback reinforces CDK2 activation and transition into S-phase. Signaling pathways interface with the core cell cycle control circuit to trigger or halt the activation of the aforementioned positive feedback loops, in part through production of G1 Cyclins and CDK inhibitors.

Cellular signaling interfaces with the core cell cycle machinery to either trigger or halt cell cycle transitions (Fig. 1.2). Growth factor signaling leads to transcriptional induction of Cyclin D, the accumulation of which kicks-off the G1-S transition [29]. On the other hand, anti-proliferative signals, such as contact inhibition and DNA damage induce the production of CDK inhibitors [30, 31], thereby preventing the onset of S-phase entry. It is unclear how cells integrate quantitative information about these signals to either remain arrested or commit to replicate DNA and divide. Moreover, how fluctuations in signaling affect the stability of established cellular states (cycling or arrested), and the conditions under which noise in signaling is buffered or amplified into changes in cellular state are not fully understood.
1.3 Processing the DNA damage signal

The genome is constantly challenged by perturbations that can compromise its physical and chemical integrity [3]. Such perturbations can be of intracellular origin, such as physical stress during DNA replication and interaction with reactive oxygen species, and they can also be of environmental origin, as it is the case of exposure to ionizing radiation, such as γ-irradiation and x-rays. Failure in the proper resolution of DNA damage can lead to genomic instability and gross chromosomal abnormalities [32, 33]. It is therefore not surprising that cells have evolved surveillance mechanisms to cope with DNA damage. In mammalian cells, detection of DNA damage results in the activation of cellular responses devoted to damage repair, the establishment cell cycle arrest, and the induction of long-term anti-proliferative programs, such as programmed cell death and cellular senescence [34, 35]. Therefore, the ability of cells to sense the presence of DNA damage constitutes both a pro-survival stress response system and an anti-proliferative tumor suppressive mechanism.

Many signaling proteins link DNA damage sensing and the activation of downstream cellular responses [36]. Among these, p53 is regarded as a master transcriptional regulator of the response of mammalian cells to DNA damage [37]. p53 has received wide attention due to its prominent role in tumor suppression [38, 39]: both homozygous and heterozygous knockout of p53 dramatically increase cancer incidence in genetically engineered mouse models [40–42]; germline mutations in p53 give place to Li-Fraumeni syndrome, which predisposes carriers to cancer development throughout life [43], and genomic surveys of human cancers revealed that the majority of tumors harbor mutations that either directly or indirectly inactivate the p53 function [44, 45]. The tight link between p53 inactivation and the development of cancer
Under unstressed conditions, p53 is constitutively produced and maintained at low basal levels due to the activity of its downstream target, the E3 ubiquitin ligase MDM2, which targets p53 to proteasomal-dependent degradation [47, 48]. Upon DNA damage, recruitment of sensory kinases (ATM, ATR) to break sites triggers a phosphorylation cascade that leads to post-translational modification of p53, resulting in relief from MDM2 dependent degradation [49, 50]. Upon accumulation, p53 orchestrates the transcriptional activation of hundreds of target genes involved in DNA repair, cell cycle arrest and apoptosis [51, 52]. Among these targets, the CDK inhibitor p21 is regarded as a major effector of cell cycle arrest upon DNA damage [19, 20, 53] (Fig. 1.3: p53 signaling links sensors and effectors in the response of human cells to DNA damage. Upon double-stranded DNA breaks, ATM is recruited to damaged sites and triggers a phosphorylation cascade that leads to p53 stabilization through the disruption of its interaction with the E3 ubiquitin ligase MDM2. p53 accumulates and orchestrates the transcriptional activation of hundreds of genes involved in the response of human cells to DNA damage. Among these target genes, the CDK inhibitor p21 directly enforces cell cycle arrest through direct inhibition of CDK activity. have prompted intense research aimed at understanding the mechanisms by means of which DNA damage and other types of cellular stress trigger p53 activation and its downstream consequences [46].
Elucidation of the aforementioned biochemical interactions has deepened our understanding of the mechanistic basis underlying the establishment of cell cycle arrest in response to DNA damage, and the way this response mechanism goes awry during malignant transformation of human cells. However, our knowledge of the perceptual capabilities of mammalian cells in the context of their response to DNA damage is more limited. Specifically, it is not clear how reliably cells translate information about the amount and duration of DNA damage exposure into downstream activation of effector proteins, such as p21. Furthermore, while perturbation-based studies have revealed that increasing p53 and p21 levels also increase the fraction of cells undergoing cell cycle arrest [54, 55], it is not clear how levels of these two proteins relate to the establishment and maintenance of this cellular state during the actual cellular response to DNA damage; furthermore, it is not known how temporal fluctuations in the level of these proteins affect the long-term maintenance of the cell cycle arrest. Such insights require detailed quantitative information regarding input-output relationships at many layers of DNA damage signal processing, critically including phenotypic outcomes.

The DNA damage response is intrinsically a dynamic system. Upon exposure to ionizing radiation, each cell receives multiple double-stranded DNA breaks. Breaks that can be repaired are resolved with heterogeneous timescales, which range from minutes to days [56], during which additional DNA damage can be spontaneously generated. Therefore, in responding to DNA damage cells are challenged with keeping track of an ever-changing signal. It is unclear the extent to which cells integrate quantitative information about persistence of DNA damage, or whether the cells rely on moment-to-moment measurements to exert responses.
Recent advances in fluorescent live cell imaging of mammalian cells have made it possible to gain insights into the quantitative and dynamic characters of signal transduction and their relationship with cell fate decision-making. Using fluorescent protein tags, the level and activity of key molecular players in well characterized signaling axes have been quantified with high temporal resolution at the single cell level [57–60]. Such efforts have revealed pervasive heterogeneity, both between cells in isogenic populations, and within an individual cell over time. One potential source of such cell-to-cell variability is at the level of signals themselves; a prime example of this is the fact that individual cells exposed to the same average dose of ionizing radiation will effectively receive variable number of double-stranded DNA breaks. However, even when the input into a signaling system remains constant, variability in responses can arise as a result of different initial states that cells populate, such as cell cycle or differentiation stage [61]. Moreover, cells that populate the same initial state and are exposed to the exact same signal can exhibit different responses due to the stochastic nature of the biochemical reactions that govern the production and degradation of individual molecules [62, 63]. Variability is therefore an intrinsic property of signaling systems that cells (and more recently researchers) have evolved to both harness and cope with.

Temporal and cell-to-cell heterogeneity in signal transduction presents both challenges and opportunities for investigators. In acknowledging the presence of substantial variation in molecular or phenotypic variables, there is a tacit acknowledgment of the limited interpretability of classical assays that rely on bulk averaging of cellular populations. This is perhaps most clearly exemplified in a situation where the levels of
a hypothetical molecule are compared between two cell populations: while in the first population this molecule varies continuously such that most cells have an intermediate level, the second group of cells is composed of a mixture of two cell types, one of which has low levels and another which has high levels of this molecule. The average number of molecules in the two cell populations is the same, but the underlying single cell distributions are drastically different. A further step in this line of reasoning is the realization that different processes can look similar under a static description even when the underlying dynamics are drastically distinct; specifically, the same distribution can be generated by both ergodic and non-ergodic processes, by dynamical systems that fluctuate with heterogeneous timescales, or by unsynchronized pulsatile vs non-pulsatile dynamics. All together, in order to distinguish between these alternative mechanistic scenarios, researchers a detailed characterization of single cell distributions and the dynamics of the underlying process that generate such distributions are often necessary.

The payoff for collecting detailed single cell and dynamical data is the opportunity to gather novel insights into the inner workings of cells. Theoretical work has suggested ways in which properties of single cell distributions beyond the average can be used to discriminate between competing hypotheses of the underlying mechanism that generates single cell distributions [64, 65]. Pre-existing heterogeneity in cell populations can predict and explain variability in the cellular response to perturbations [66–68], and statistical relationships between molecular and phenotypic variables within the same cell have been used to uncover interactions within signaling pathways [69, 70]. In a particular instantiation of this approach, heterogeneity can be leveraged to decompose cell populations into subpopulations with similar phenotypes; since all cells are theoretically exposed to equivalent environmental and experimental conditions,
these sources of measurement variability are conditioned out when comparing between groups within the same population. An extension of this rationale is the comparison of individual cells within cellular lineages, an approach that can help determine the relative contribution of genetic and epigenetic history to heterogeneity in molecular and phenotypic traits [71]. Lastly, by recording the whole history of a dynamical process it is possible to study infrequent or unsynchronized events, which are hard to observe with static assays or population averaging. This diversity in approaches attests to the way our ability to query dynamical processes at the single cell level has motivated the development of analytical tools aimed to maximize the insights gained from such experiments.

While surveys of molecular and phenotypic forms of cell-to-cell variation represent a relatively recent scientific development, such variability is an integral part of the biology of cells and their evolution [72]. There is a lot of interest to determine the conditions under which noise in signaling limits biological function, and the situations under which noise itself confers properties that would be difficult to achieve otherwise. Theoretical work has pointed out that noise imposes hard limits to cellular control circuits and signal transduction cascades [73]. On the other hand, it has also been proposed that high variance is a property of molecules that carry information within cells, and that fluctuations can enhance, rather than limit, the function homeostatic control [74]. Noise has also received wide attention as a source of phenotypic diversity in isogenic cell populations. In specific instances, such phenotypic diversity has been linked with clinically relevant phenotypes, such as resistance to chemotherapy [66, 75, 76] and antibiotics [77]. Furthermore, noise-driven destabilization of the pluripotency maintenance circuitry has been proposed as a trigger of embryonic stem cell differentiation [56]. Recently, noise in the levels of a DNA repair enzyme in bac-
Figure 1.4: Insights gained from quantifying p53 protein dynamics in individual cells. A. p53 shows pulsatile dynamics after ionizing radiation. p53 transcriptionally activates production of the E3 ubiquitin ligase MDM2; in turn, MDM2 tags p53 for proteosome dependent degradation, forming a negative feedback loop. B. p53 shows stimulus dependent activation. While ionizing radiation gives place to pulsatile p53 dynamics, UV radiation gives place to a single, broad pulse of p53. This difference in dynamics was instrumental to identify stimulus dependent interactions in the p53 feedback control circuit. C. Stabilization of p53 using the small molecule nutlin-3a switches p53 dynamics from pulsatile to sustained in response to γ-irradiation. As a result of this perturbation, cells lose recovery potential and enter a state of cellular senescence.
the form of periodic pulses with stereotyped duration and variable amplitude [80, 81] (Fig. 1.4A). This observation stands in contrast with previous work that quantified p53 dynamics using western blots, which described dampening of p53 oscillations in response to ionizing radiation [82]. The difference between single cell and population level data can be reconciled when taking into account the effect of de-synchronization and averaging of discrete pulses that are observed in individual cells. Pulsatile p53 dynamics were informative in constraining quantitative models of the molecular circuitry that controls p53 activation; beyond MDM2 dependent degradation, computational modeling revealed the relevance of other sources of negative feedback regulation of p53 that lead to recurrent pulsatile behavior [83] (Fig. 1.4B). Furthermore, quantification of p53 dynamics in individual cells has been used to characterize the heterogeneity in DNA damage signaling in response to chemotherapy and the way cell-to-cell variation at the molecular level relates to fractional killing of cancer cells [75, 84].

A particular question stemming from the observation of p53 pulses was whether this dynamical pattern was in any way important for the cellular response to ionizing radiation. Using a small molecule that inhibits the interaction between p53 and MDM2, it was possible to re-shape p53 activation from pulsatile to sustained dynamics. The outcome of this perturbation was a markedly reduced recovery of cell and entry into a phenotypic state known as cellular senescence [85] (Fig. 1.4C, discussed in detail below). Recently a similar approach has been used to show that transient stabilization of p53 during G2 is sufficient to trigger cytokinesis failure and entry into G1 in a tetraploid state, with cellular senescence as a long-term outcome [55, 86]. These two observations mark a precedent for the importance of quantitative regulation of p53 levels and the way subtle and transient perturbations to the system can bring
about large differences in fate, long after exposure to DNA damage.

1.5 The long-term phenotypic consequences of DNA damage

Over long-timescales, exposure to ionizing radiation can lead to distinct, possibly co-existing physiological states: cells can either recover and resume proliferation, or activate anti-proliferative programs, such as programmed cell death or cellular senescence. Cellular senescence is characterized by the long-term enforcement of cell cycle arrest and the loss of recovery potential [87]. Recognition of cellular senescence as a phenotypic state goes back to early attempts to establish in vitro cultures of non-transformed cells. In 1961, Hayflick and Moorehead reported conclusive evidence that primary human fibroblasts have limited replicative capacity, which was attributed to a cell intrinsic countdown division counter [88]. Over 50 years later, we know that this counter can be mapped to the progressive erosion of chromosome ends known as telomeres, which results as a natural consequence of the mechanism by way of which cells replicate linear DNA [89], a phenomenon known as the end-replication problem. Moreover, we know that a multiple proteins assemble at chromosome ends and prevent them from being recognized as double-stranded DNA breaks. Progressive shortening of telomeres [90] eventually leads to de-protection, the activation of the DNA damage response and the consequent enforcement of cell cycle arrest [91]. In stem cells, an RNA-protein complex named telomerase elongates chromosome ends and contributes to the extension of replicative capacity [92]. Telomerase overexpression is common in cancer cells [93], and also serves as a tool to immortalize primary human cells for research purposes [94].

Diverse forms of cellular stress trigger cellular senescence, including DNA damage
[95, 96], telomere erosion [91] and oncogenic activation [97]. Senescent cells have been identified in pre-tumorigenic lesions and are absent in their malignant counterparts; therefore, cellular senescence is regarded as potent tumor suppressive mechanism that cells either bypass or escape in their route to malignant transformation [98]. Moreover, the establishment and clearance of senescent cells have been implicated in the response of tumors to cytotoxic chemotherapy, highlighting the role of this phenotypic state in the context of cancer treatment [99, 100]. In the literature senescence is described as an irreversible form of cell cycle arrest that stands in contrast to the reversible quiescent state; however, throughout the years it has also been reported that senescent cells can spontaneously escape the arrested state [101] and with low probability re-gain the capacity to form colonies [102]. Full reversal of the senescent phenotype is associated with genomic instability and silencing of tumor suppressors at the time colonies become visible. It is not clear how the transition from a stably arrested state back to a proliferating state initially happens.

1.6 A ROADMAP FOR THIS THESIS

The long-term response of human cells to ionizing radiation has been extensively studied using bulk population and static assays. These approaches have been pivotal for our understanding of the molecular circuitry that mediates cell cycle arrest and senescence upon DNA damage; however, they lack the resolution to address questions concerning temporal and cell-to-cell variation in the processes of cell cycle arrest establishment, maintenance and exit. To gain an understanding of such heterogeneity, we used live-cell imaging to quantify the number and timing of division events that individual human cells go through in the course of one week after exposure to ionizing radiation.
Variability was evident among senescing cells, in terms of the maintenance of the arrested state, and among recovering cells, in terms of the timescale of cell cycle re-entry. In Chapter 2, we describe our efforts to understand the mechanistic basis of variability in the long-term maintenance of cell cycle arrest in damaged cells. Using fluorescent reporters of key molecular players in DNA damage signaling, we identified a situation under which noise in signaling drives cell cycle re-entry in long-term arrested cells. In Chapter 3, we study the consequences of heterogeneity in recovery timescales in the context of combination cancer therapy. We revealed that combined treatment of human cells with ionizing radiation and the Eg5 kinesin inhibitor STLC, which selectively targets mitotic cells, leads a qualitative re-shaping of the DNA damage dose response curve, where an intermediate level of radiation optimizes cell proliferation. Finally, in Chapter 4 we propose a continuum model of cell fate in the response of human cells to ionizing radiation and revisit long-standing questions in DNA damage signaling in light of this model. By unmasking such continuum in cellular phenotypes, our work provides a framework to conceptualize heterogeneity in cell fate in terms of variability in arrest duration and recovery potential. A quantitative account of the dynamics of DNA damage signaling will be pivotal for our understanding of the temporal regulation of cell fate after ionizing radiation and the way genetic and pharmacological interventions can modulate arrest-exit time distributions.
Noise driven escape from cell cycle arrest

Biological signals need to be robust, and filter small fluctuations yet maintain sensitivity to signals across a wide range of magnitudes. Here we studied how fluctuations in DNA damage signaling relate to maintenance of long-term cell cycle arrest. Using live-cell imaging, we quantified division profiles of individual human cells in the course of one week after irradiation. We found a subset of cells that initially establish cell cycle arrest, and then sporadically escape and divide. Using fluorescent reporters and mathematical modeling, we determined that fluctuations in the oscillatory pattern of the tumor suppressor p53 are amplified into a sharp switch between p21 and CDK2, leading to escape from arrest. Our results show that noise propagation and amplification can translate small fluctuations into large phenotypic changes.
Figure 2.1: Diverse cellular phenotypes can result from exposure of human cells to ionizing radiation. A. DNA damage can lead to different cellular outcomes, including terminal cell fates. Cellular senescence requires active maintenance. B. Representative images of cells assayed for senescence associated β-galactosidase (SA-β-gal) activity 6 days post-irradiation. C. Frequency of SA-β-gal positive cells 6 days post-irradiation, as a function of damage dose.

In response to DNA damage, proliferating cells can either repair the damage and resume growth, or activate anti-proliferative programs such as cell death (apoptosis) or senescence, a state characterized by the long-term enforcement of cell cycle arrest and the loss of recovery potential (Fig. 2.1A). Pro-apoptosis therapy has been used for several decades as a tool for destroying the growth of cancerous cells, while recent studies also highlighted the therapeutic potential of pro-senescence cancer therapy [98, 100, 103]. However, as opposed to apoptosis, which is a terminal cell fate, senescing cells require continuous activation of the pathways responsible for maintaining the arrested state (Fig. 2.1A). It is unclear how senescing cells respond to fluctuations in these pathways over prolonged times.
2.1 Individual cells exhibit heterogeneity in the long-term maintenance of cell cycle arrest

We used fluorescent live cell imaging to study DNA damage-induced senescence in individual human cells. We irradiated cells and developed a semi-automated tracking method to quantify the number and timing of division events that an isogenic population of cells experience in the course of one week, during which they undergo senescence [55] (Fig. 2.1B, C). Division profiles revealed large heterogeneity between single cells, ranging from cells that did not divide at all, through cells that divided only once (single dividers Fig. 2.2 red squares), and cells that showed continuous divisions (Fig. 2.2), the proportion of which changed with irradiation dose (Fig. 2.2).

Single dividers exhibited bimodality in their mitosis timing: the first phase of divisions occurred within the first day after DNA damage, and the second phase started 2 days post-irradiation and was broadly distributed across the entire week (Fig. 2.3A). Using the mVenus-hGeminin(1-110) fluorescent reporter for cell cycle progression [104] (Fig. 2.3B), we found that heterogeneity in the second phase is mainly attributed to time spent in G1 state (Fig. 2.3C, D), suggesting that late dividing cells initially arrested in G1 and then escaped from the arrest.

Using a fluorescently tagged 53bp1 protein [56, 105] (Fig. 2.4A, B) we quantified DNA damage in live cells and found that the late dividers undergo G1-S transitions in the presence of DNA damage. Specifically, they exhibited a higher number of 53bp1 foci than those of unirradiated cells, and similar to those of irradiated non-dividing cells (Fig. 2.4C, D), strengthening the suggestion that these late divisions (Fig. 2.2)
Figure 2.2: DNA damage leads to heterogeneous division profiles over long timescales. Division profiles obtained after tracking individual telomerase-immortalized primary cells and annotating mitoses in the course of one week after DNA damage. Panels aggregate single cells exposed to a particular irradiation dose. Each row represents the division profile of an individual cell over time. Colors change upon mitosis. Cells are grouped by their total number of mitoses, and ordered by the timing of their first mitosis. Red boxes highlight the single divider populations.
Figure 2.3: A subpopulation of cells escape G1 arrest over long timescales. A. Distribution of mitosis timing in single dividers. B. Single cell quantification of mVenus-hGeminin(1-100) reporter for a multiple divider (top) and a late divider (bottom). C, D. Distributions of G1 and S/G2 duration in unirradiated cycling cells or irradiated late dividers.
are not a consequence of complete repair, but rather reflect a limit on the ability of cells to maintain cell cycle arrest in the presence of damage. We termed these late dividing cells ‘escapers’.

2.2 **Cell-to-cell variability in DNA damage signaling contributes to escape from arrest**

We next sought to determine whether escape events can be explained by levels of the molecular players that control cell cycle arrest. The p53 signaling pathway links DNA damage sensing with cell cycle arrest [37] (Fig. 2.5A). Upon irradiation, the tumor suppressor protein p53 transcriptionally activates hundreds of genes involved in DNA repair, cell cycle arrest and apoptosis [51]. One of such target genes, the Cyclin Dependent Kinase (CDK) inhibitor p21 [53], directly enforces G1 arrest through sequestration of Cyclin-CDK complexes [18, 19, 106]. Both p53 and p21 are necessary to establish cell cycle arrest after damage in our experimental system (Fig. 2.5B). Note that knockdown of p53 either 2 days or 7 days post-irradiation triggered cell cycle re-entry in arrested cells (Fig. 2.5C), demonstrating the important role that p53 signaling plays for both the establishment and maintenance of the arrested state.

To quantitatively measure the relationship between p53, p21 and escape from arrest in individual cells we established a cell line harboring a p53 fluorescent fusion protein [80] and applied a recently developed CRISPR-Cas9 based method [107] to endogenously tag p21 with the fluorescent protein mKate2 (Fig. 2.5D,E). After irradiation, we observed that oscillatory p53 dynamics, which were previously described during the first 48h post-irradiation [80], persisted for the entire experimental time
Figure 2.4: Cells escape G1 arrest in the presence of DNA damage. A. Cells expressing mCherry-53bp1 were exposed to 10Gy gamma irradiation. DNA damage leads to foci of mCherry-53bp1 (2h post-irradiation); over time the number of foci decreases (24h post-irradiation). B. To assess the reliability of mCherry-53bp1 as a marker of persistent DNA damage foci, cells were immunostained for the canonical DNA damage marker phospho-γ-H2A.X. Four representative cells showing co-localization of mCherry-53bp1 and γ-H2AX are shown. C. Cells received 20Gy gamma irradiation and were imaged for 48h, 4 days after DNA damage. Image strips of mCherry-53bp1 and mVenus-geminin(1-100) are shown for an individual cell tracked through an arrest escape event, as evidenced by the onset of mVenus-hGeminin accumulation (red dashed line). mCherry-53bp1 shows the presence of DNA damage prior to escape from cell cycle arrest. D. Distribution of the number of 53bp1 foci in non-irradiated cells in G1, irradiated escaper cells and cells that maintain cell cycle arrest in the timeframe of the experiment.
Figure 2.5: Fluorescent tagging allows quantification of p53 and p21 dynamics in live individual cells. A. In response to DNA damage p53 transcriptionally activates p21 which arrest the cell cycle. B. Fraction of cells that divide after siRNA mediated depletion of p53 or p21, 2 days post-DNA damage (20Gy). *** p < 0.001, two-tailed binomial proportion test. C. siRNA mediated depletion of p53 at 2 days or 7 days post-DNA damage leads to cell cycle re-entry. *** p < 0.001, two-tailed binomial proportion test. D. A fluorescent fusion protein and endogenous tagging allows quantification of p53 and p21 dynamics in individual cells. E. Western blot confirms heterozygous endogenous tagging of p21 protein with the fluorescent protein mKate2 in two independently derived clones. Clone 3F is used to simultaneously quantify p53 and p21 dynamics. Clone 10 is used in conjunction with CDK2 activity reporter DHB-mVenus (see below). F. Representative p53 and p21 trajectories of a single cell exposed to 10Gy γ-irradiation. G. Trajectories of p53 and p21 derivatives calculated from traces shown in (E). Changes in p21 protein closely follow changes in p53 with a time delay.
(5 days) (Fig. 2.5F). Following p53 activation, p21 levels increased (Fig. 2.5F) with dynamics that inherited the upstream p53 oscillations and with a time delay of 3h, as evidenced by cross-correlation between p53 and p21 derivative trajectories (Fig. 2.5G).

In agreement with studies on single cell p53 dynamics in other cell lines [108, 109], p53 oscillations had a stereotyped period of 5.5h (Fig. 2.6A) and noisy amplitude (Fig. 2.5F) which was previously attributed to arise from intrinsic fluctuations in the feedback control circuit that generates p53 pulses [108]. The period of p53 oscillations was similar between non-dividing and escaper cells (Fig. 2.6A); however, escapers fluctuated around a lower averaged amplitude of p53 compared to non-dividers (Fig. 2.6B). Similar behavior was observed for the averaged levels of p21 (Fig. 2.6C), which correlated with p53 levels at the single cell level (Fig. 2.6D). Consistently, we were able to increase the percentage of cell cycle re-entry of arrested cells by partial siRNA-based knockdown of p53 (Fig. 2.6E, F). Taken together, our results show that cell-to-cell variability in the levels of p53 and p21 contribute to the heterogeneity observed in the long-term maintenance of cell cycle arrest after irradiation, with escapers having overall lower averaged levels of p53 and p21 than non-dividing cells.

2.3 Escape from arrest is characterized by a sharp switch in p21 levels and CDK2 activity

While the distributions of p53 and p21 differ significantly between non-dividers and escapers there was an extensive overlap between them (Fig. 2.6B, C), suggesting that properties of p53 and p21 trajectories beyond average levels may be linked to the es-
Figure 2.6: Cell-to-cell variation in DNA damage signaling contributes to heterogeneity in arrest maintenance. **A.** Autocorrelation functions estimated from p53 trajectories of non-dividers or escapers. Bold lines and shaded areas correspond to median and interquartile range, respectively. **B, C.** Distributions of mean p53 pulse amplitude (B) and mean p21-mKate2 intensity (C) after γ-irradiation in escapers and non-dividers. **D.** Correlation of mean p53 pulse amplitude and mean p21-mKate2 intensity. Each dot represents a single cell. **E.** Distributions of p53-mNeonGreen intensity in p53 (4nM and 32pM) or control siRNA treated cells, 3 days post-irradiation (20Gy). **F.** Fraction of cells that divide within 2 days after siRNA knockdown (E). *** p < 0.001, two-tailed binomial proportion test.
cape from arrest. To further understand the series of molecular events leading to arrest escape, we followed p21 dynamics in individual cells several hours before cell cycle re-entry. While non-dividers maintained sustained p21 levels through the entire duration of the experiment (Fig. 2.7A), p21 was sharply degraded upon escape from G1 arrest and S-phase entry (Fig. 2.7B).

p21 halts cell cycle progression through direct inhibition of Cyclin-CDK complexes. The CyclinE-CDK2 complex in turn drives transition into S-phase and leads to p21 degradation, forming a double negative feedback loop [22] (Fig. 2.7C). Using a recently established translocation based fluorescent reporter of CDK2 activity [110] (Fig. 2.7D, E), we found that the onset of sharp p21 degradation was tightly correlated with an increase in CDK2 activity, leading to a switch in the abundance and activity of these two molecular players (Fig. 2.7F). These findings suggest that, while elevated p21 in escapers is sufficient to maintain the arrested state for several days, cells experience an event that triggers the switch between p21 and CDK2. We sought to determine the molecular mechanism that triggers such an abrupt switch.

2.4 Fluctuations in p53 are amplified into escape from arrest

The interplay between p21 and CDK2 can function as a bistable toggle switch in a deterministic dynamics framework [111]. To understand whether and how this core circuit might be affected by temporal fluctuations stemming from p53, we adapted a previously developed computational model that accounts for deterministic dynamics of p21 and CDK2 interactions [24], and introduced pulsatile p53 dynamics as the only source of variation in p21 production (Fig. 2.8A). Our model showed that sequences
Figure 2.7: Escape from cell cycle arrest is characterized by a sharp switch in the balance between p21 and CDK2 activity. A, B. Representative single cell trajectories of p21-mKate2 levels in irradiated non-dividers and escapers. Vertical lines denote mitosis events. C. p21 arrests the cell cycle by inhibiting the function of the CDK2/CyclinE complex which in turns leads to p21 degradation upon S-phase entry, forming a double negative feedback loop. D. CyclinE-CDK2 dependent phosphorylation triggers cytoplasmic retention of DHB-mVenus, which serves as a quantitative proxy for CDK2 activity in live cells. E. Representative images of cells harboring CDK2 activity reporter and p21-mKate2 fluorescent protein. Nuclei are outlined in orange. F. Dynamics of p21 and CDK2 activity in the vicinity of escape events. Irradiated cells were in silico synchronized to the time of p21 degradation. Bold lines and shaded areas correspond to median and interquartile ranges, respectively.
Figure 2.8: Double-negative feedback between p21 and CDK2 can act as a non-linear amplifier of upstream p53 fluctuations. 

**A.** Schematic of our computational modeling approach. p53 trajectories are generated by randomly sampling pulse amplitude values, and represent the input into a deterministic ODE based model of p21 and CDK2 (Overton, et al. 2014).

**B.** Representative simulation result capturing escape from cell cycle arrest. 

**C.** Simulated p53 trajectories were in silico aligned to the time of p21 degradation. Random non-dividing cells were sampled to match the timescale of escape events. Median trajectories +/- interquartile range are compared in the vicinity of G1-S transitions.

**D.** p53 and p21 dynamics were quantified in the same cell. Three representative single cell trajectories of escapers are shown. 

**E.** p53 trajectories were in silico synchronized to the time of p21 degradation for escapers. Trajectories from non-dividers were randomly sampled to match the time of escape events. Bold line and shaded area correspond to median and interquartile range, respectively. *** p < 0.001, two tailed t-test. Escapers show overall lower levels of p53 than non-dividers, and a dip in p53 pulse amplitude prior to the p21/CDK2 switch that leads to the escape from arrest.
of low amplitude p53 pulses preceded sporadic events of p21 degradation (Fig. 2.8B, C), suggesting that the double negative feedback between p21 and CDK2 can amplify fluctuations in p53 pulses and trigger escape from arrest.

To test whether local fluctuations in p53 pulse amplitude indeed contribute to escape from cell cycle arrest, we quantified p53 dynamics in >1000 individual cells that either maintained or escaped the arrested state in the course of 5 days post-irradiation (Fig. 2.8D). We used p21 degradation events to in silico align individual cells at the time of cell cycle re-entry, allowing a retrospective view at p53 fluctuations in the vicinity of G1-S transitions. We found that a transient decrease in p53 pulse amplitude preceded the onset of p21 degradation events, a pattern that was absent in trajectories from non-dividers randomly sampled to match the timing of unsynchronized escape events (Fig. 2.8E). Our data supports a model in which quantitative variation in p53 pulse amplitude defines a permissive state within which noise propagation and amplification drives cell cycle transitions (Fig. 2.9). Specifically, low average p53 pulse amplitude primes cells into a cellular state in which transient fluctuations in p53 are amplified into escape from cell cycle arrest. Therefore, both the average and tails of the p53 distribution contribute to the noise-driven cell cycle transitions of individual cells.

Escape from G1 arrest is associated with abnormal mitoses and errors in chromosome segregation. Micronuclei formation, which occurs when lagging or fragmented chromosomes fail to be incorporated into the main nucleus after mitosis (Fig. 2.8A), is more frequent in escapers (single dividers) than in multiple divider cells (Fig. 2.8B). Moreover, such mitosis events are followed by induction of p53 and p21 to higher levels than those before escape from arrest, and a shift from pulsatile to sustained p53
dynamics (Fig. 2.8C–E). Collectively, chromosome missegregation and potentiation of DNA damage signaling may account for the lack of cell division observed after escape from G1 arrest in the single divider population. Abnormal ploidy is known to have general deleterious effects both in non-transformed and cancer cells, and changes in ploidy can trigger cell cycle arrest and senescence even in the absence of DNA damage [112]. We note, however, that chromosome missegregation is also associated with the genomic instability characteristic of solid tumors [33, 113] and it is possible that the phenotypic plasticity generated by fluctuations in DNA damage signaling could precede the establishment of rare clones that hold the potential to revert the senescent phenotype and resume active proliferation as a result of a particular chromosomal configuration [102].

Whether cellular noise is beneficial for cells or an obstacle they need to buffer and cope with is an unsolved question. On one hand, noise was shown to impose limits to cellular control circuits [73], and cells have evolved mechanisms to distinguish bona fide signals from noise [114]. On the other hand, fluctuations in signaling have been functionally linked to the generation of phenotypic diversity that shapes multicellular development [27] and allows cells to adapt to changes in their environment [66, 77].
In the context of p53 signaling, noise driven escape from arrest may be the reflection of a trade-off between the need of cells to respond robustly to low levels of damage while also maintaining a recovery potential [85]. Since maintenance of cell cycle arrest is critical for the success of cytotoxic chemotherapy [98, 100], it will be important to further understand the extent to which noise driven cell cycle re-entry can facilitate early stages of senescence escape and resistance to cancer therapy.

In our system, the temporal fluctuations in p53 signaling were shown to drive large phenotypic changes when integrated and amplified by downstream molecular circuits. A similar noise driven switch has also been shown in Bacillus subtilis, in which an excitable system of negative and positive feedback loop led to transient differentiation [116]. The function of noise and its role in switching cellular states in other mammalian systems is still unclear. Careful quantitative measurements in single cells will be required to determine whether other systems show similar responses to fluctuations, and specifically whether cells can more efficiently filter fluctuations in oscillatory systems than non-oscillatory systems, giving some hints to the puzzling function of oscillations in transcriptional regulation.

2.5 Materials and Methods

Materials

Cell Culture

RPE cells immortalized with telomerase overexpression (RPE-hTERT, a kind gift from S.J. Elledge, Harvard Medical School) were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, 100mg/mL streptomycin
Figure 2.10: Escape from arrest is associated with chromosome missegregation and re-enforcement of DNA damage signaling. A. Representative images of escaper cell before and after mitosis. H2B-mTurquoise physically associates with DNA in the nuclear compartment and micronuclei (white arrows). B. Frequency of cells born with micronuclei as a function of irradiation dose and cell fate (3+ divisions for multiple dividers or 1 division in the case of escapers). Only first mitosis event is taken into account. C. Representative single cell trajectories of escaper cells that divided close to 4 days post-irradiation. p53 is induced to higher levels after mitosis. D, E. Median (bold line) and interquartile range (shaded area) p53 and p21 levels in escaper cells aligned at time of mitosis. Non-divider cells were randomly sampled to match escaper mitosis timing. While p53 and p21 remain within the same range during the sampled time, p53 and p21 are re-induced into higher levels post-mitosis.
and 250ng/mL fungizone (Gemini Bio-Products). For microscopy, DMEM/F12 + 5% FBS lacking phenol red and riboflavin, was used. When necessary, media was supplemented with selective antibiotics (400μg/mL G418, 2μg/mL puromycin).

To endogenously tag p21 with the fluorescent protein mKate2, we used the eFlut toolset as previously described (Stewart-Ornstein, 2016). The resulting tagged coding sequence includes a P2A cleavable Neomycin resistance protein, allowing selection for cells harboring the tag without disrupting the endogenous p21 3'UTR. To establish the p21-mKate2/p53-mNeonGreen dual reporter cell line, p21-mKate2 cells were infected with a p53-mNeonGreen lentiviral vector. Single cell clones were expanded through limited dilution and subsequently screened for marker expression. To establish the p21-mKate2/DHB-mVenus reporter line, clonal p21-mKate2 tagged cells were infected with the DHB-mVenus lentiviral vector (a kind gift from S. Spencer, University of Colorado Boulder, Spencer et al. 2012). Cells were further infected with mCerulean3-NLS lentiviral vector, a constitutive nuclear marker.

**Irradiation**

Cells were exposed to time-controlled doses of γ-irradiation in a ⁶⁰Co irradiator.

**Antibodies and Reagents**

Primary antibodies for p21 (Ab1, Calbiochem), RFP (PM005, MBL international) and H2A.X-P(Ser139) (JBW301, Millipore) were used at 1:1000-1:2000 dilutions for Western Blot, and at 1:100 dilution for immunofluorescence. Secondary goat anti-mouse antibody, conjugated to AF488 was purchased from Invitrogen; secondary goat anti-mouse antibodies coupled to IR800 or IR680 were purchased from LI-COR Biosciences. DAPI was purchased from Life Technologies and used at a 1:104 dilu-
tion. Pooled small interfering RNA (siRNA) targeting p53, p21 or control siRNA were purchased from Dharmacon, and delivered to cells using RNAiMAX reagent (Thermo Fisher Scientific).

**Methods**

**Live-Cell Microscopy**

Cells were grown in poly-D-lysine-coated glass bottom plates (MatTek Corporation) and imaged using a Nikon Eclipse TE-2000 inverted microscope with a 10X Plan Apo objective and a Hammamatsu Orca ER camera, equipped with environmental chamber controlling temperature, atmosphere (5% CO2) and humidity. For long-term live cell imaging experiments (>5 days), media was replaced every day to maintain growth factor stimulation throughout the entire duration of imaging. Images were acquired every 30 min using the MetaMorph Software.

**Single Cell Tracking and quantification**

A semi-automated method was developed that allows tracking and cell fate annotation of individual, fast moving cells over long timescales. Our method relies on (i) automatic identification of single cell centroids using intensity and shape information of a constitutive nuclear marker; (ii) centroid linkage and track propagation using nearest-neighbor criteria; and (iii) real-time user correction of tracking, and annotation of cell fate events. Tracking data was then used to quantify intensity of fluorescent reporters from background subtracted images by averaging 10 pixels within the cell nucleus. In order to quantify cytoplasmic-nuclear ratio of fluorescence intensity, cytoplasmic signal was estimated by averaging the intensity of a 3 pixels-wide ring surrounding segmented nuclei, which was then divided by the average nuclear intensity.
Matlab scripts for tracking and quantification of single cell data are available upon request.

**siRNA knockdown**

Small interfering RNA (siRNA) targeting p53, p21 or control siRNA were delivered to irradiated cells using RNAiMAX reagent (Thermo Fisher Scientific), following manufacturer protocol. Media was replaced 5h post-delivery, and cells were imaged for >2 days to quantify cell cycle re-entry. Live reporters allowed confirmation of target knockdown.

**Mathematical modeling**

Computational analysis of the effect of fluctuations in p53 pulse amplitude on the core p21-CDK2 double negative feedback loop was performed using ode45 solver in Matlab (Mathworks). For detailed description of modeling approach see Supplementary Text below.

**Senescence Associated β-Galactosidase Assay**

Senescence Associated β-Galactosidase activity was assayed using the corresponding staining kit purchased from Cell Signaling (cat#9860), following manufacturer protocol. Cells received γ-irradiation, replated 3 days post-irradiation adjusting for differences in cell density associated with different damage doses, and stained for β-Galactosidase activity. Cells were imaged using a Nikon Eclipse TE-2000 inverted microscope equipped with an IDEA digital color camera and a 10X Plan Apo objective.

**Immunofluorescence**

Cells were plated in poly-D-lysine coated glass bottom dishes (MatTek Corpora-
tion) and fixed with 4% paraformaldehyde for 10 min at room temperature at the appropriate time after treatment. Cells were permeabilized with 0.1% Triton-X and blocked with 2% BSA in 1X PBS. Cells were incubated overnight with primary antibody, washed, and stained with secondary fluorescent antibodies and DAPI for 1 h, followed by a final wash. Cells were imaged using a Nikon Eclipse TE-2000 inverted microscope with a 20X Plan Apo objective and a Hammamatsu Orca ER camera.

**Western Blot**

Cells were harvested with RIPA buffer (Cold Spring Harbor Protocols) containing protease and phosphatase inhibitors. Protein samples were separated by electrophoresis on 4%-12% Bis-Tris gradient gels (Invitrogen) and transferred to Pure Nitrocellulose Blotting Membrane (Life Sciences). Membranes were blocked with 4% BSA, incubated with primary antibody overnight, washed, and incubated with secondary fluorescent conjugate antibodies, followed by a last wash. Membranes were scanned using LI-COR Odyssey CLX.

**Statistical analysis of single cell trajectories**

The concurrent quantification of protein dynamics and cell fate at the single cell level allows the identification of subpopulations exhibiting distinct phenotypic behaviors, which can be then used to investigate the statistical relationship between properties of time trajectories of molecular players and cell fate. Such approach can be used to extract quantitative relationships within populations of cells that are subject to similar environments and experimental sources of variability and differ only on their fate. To compare quantitative features of p53, p21 and CDK2 that distinguish non-dividers from escapers, we consider full trajectories of non-dividers, and partial trajectories of escapers up until the time of G1-S transition. As a special case, we consider portions
of p21 trajectories in which this protein was above basal levels (induced state). Such consideration is important because cells that receive damage in S have delayed p21 induction owing to the active p21 degradation program during this cell cycle phase [22, 107].

*In silico* alignment of trajectories was used throughout this work to project cells undergoing unsynchronized escape events into comparable time axes relative to such events. Non-dividers are sampled at random using time coordinates of individual escape events, thereby constituting a null distribution in this relative time axis.

**Mathematical modeling**

We consider a deterministic model with four molecular species: p53 protein, p21 mRNA, p21 protein and active CDK2. This model consists of a simplified architecture of the core double negative feedback loop between p21 and CDK2, and is adapted from a previously develop computational model (Overton et al. 2014). The dynamics of this system are described by the following coupled ordinary differential equations:

\[
\frac{dp21_{mRNA}}{dt} = \alpha_1 p53 - \beta_1 p21_{mRNA} \tag{2.1}
\]

\[
\frac{dp21_{prot}}{dt} = \alpha_2 p21_{mRNA} - \beta_2 p21_{prot} - \beta_3 p21_{prot} \left( \frac{CDK2^{n1}}{CDK2^{n1} + K_{n1}} \right) \tag{2.2}
\]

\[
\frac{dCDK2}{dt} = \alpha_3 + \alpha_4 CDK2 - \beta_4 CDK2 - \beta_5 CDK2 \left( \frac{p21_{prot}^{n2}}{p21_{prot}^{n2} + K_{n2}} \right) \tag{2.3}
\]

The model receives p53 time trajectories with fixed period and variable amplitude. Trajectories are generated by scaling sine functions with amplitude values sampled from a log-normal distribution. Each pulse is sampled independently.
The aim of the computational model described here was to develop an intuition regarding the way fluctuations in p21 levels stemming from variable p53 oscillations could give place to stochastic switching between a $p21_{\text{high}}/\text{CDK2}_{\text{low}}$ (arrest) to a $p21_{\text{low}}/\text{CDK2}_{\text{high}}$ (escape) state. To explore the dynamical behavior of this quantitative model, we first simulated the dynamics of this system in the absence of temporal fluctuations in p53 oscillations. We then asked the extent to which introducing fluctuations in p53 pulse amplitude could drive a stochastic switch into a $p21_{\text{low}}$ state in a system that would have otherwise maintained a $p21_{\text{high}}$ state. Statistics on features of p53 trajectories in the vicinity of transitions were estimated from 5,000 simulated cells.

The following table describes parameters used in Fig 2.8. Rate constants are in nM/min.

**Table 2.1: Parameters used in computational model.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
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<tbody>
<tr>
<td>$\alpha_1$</td>
<td>p53 dependent production of p21 mRNA</td>
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</tr>
<tr>
<td>$\alpha_2$</td>
<td>p21 protein production</td>
<td>2</td>
</tr>
<tr>
<td>$\alpha_3$</td>
<td>Basal active CDK2 production</td>
<td>1</td>
</tr>
<tr>
<td>$\alpha_4$</td>
<td>CDK2 positive feedback</td>
<td>1</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>p21 mRNA degradation</td>
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</tr>
<tr>
<td>$\beta_2$</td>
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</tr>
<tr>
<td>$\beta_3$</td>
<td>CDK2 induced p21 protein degradation</td>
<td>7</td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>Basal CDK2 inactivation</td>
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<tr>
<td>$\beta_5$</td>
<td>p21 induced CDK2 inactivation</td>
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<tr>
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<td>Affinity constant for CDK2-dependent p21 inhibition</td>
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<tr>
<td>$k_2$</td>
<td>Affinity constant for p21-dependent CDK2 inhibition</td>
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<td>Hill coefficient describing ultrasensitivity of CDK2-dependent p21 inhibition</td>
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<tr>
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<tr>
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<td>Standard deviation of p53 pulse amplitude distribution (log-normal)</td>
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2.6 Acknowledgements

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2.7 Author contributions

José Reyes conducted experiments and analyzed data; Jia-Yun Chen established the mVenus-hGeminin(1-110) RPE cell line; Jacob Stewart-Ornstein generated plasmids for the establishment of p53 and p21 fluorescent reporters; José Reyes and Kyle W. Karhohs developed image analysis framework; José Reyes and Galit Lahav wrote manuscript. All authors contributed with critical experimental design suggestions.
Antagonism between anti-cancer treatments leads to non-monotonicity in the cellular response to DNA damage.

The specific outcome of combination cancer therapy depends, qualitatively, on the way treatments interact and quantitatively, on parameters such as dosing and schedules. Here we focused on the interaction between DNA damage and the Eg5 kinesin inhibitor S-Trityl-L-Cysteine (STLC), which specifically targets mitotic cells. DNA damage antagonized STLC treatment through establishment of cell cycle arrest, leading to a non-monotonic dose response curve in which an intermediate dose of damage optimized cell viability. Optimal damage dose shifted as a function of STLC treatment duration and the irradiation dose response curve could be modulated.
Figure 3.1: Ionizing radiation diversifies cellular states in isogenic cell populations. 

A. Increasing dose of x-ray leads to decreasing proliferation potential, as quantified by total number of divisions 5 days after damage. B. Within cells that divide at least once, cell cycle arrest duration increases in a DNA damage dose dependent manner. Intermediate doses of damage are characterized by high variance in the timing of first division.

through chemical and genetic perturbation of DNA damage signaling. We aim to understand the extent to which the cellular response to antagonistic cancer treatments is subject to optimization through quantitative tuning of signal transduction.

The overarching goal of cancer therapy is to selectively thwart the growth of malignant cells while minimizing damage to normal tissue. Combinatorial therapeutic regimens have been proposed as means to improve pre-existing treatments, and constitute standard of care for specific types of cancer [117]. Treatment combinations can result in distinct qualitative modes of interaction, including additivity, synergism, antagonism and suppression [118] The specific mode of interaction has important consequences on the long-term outcome of treatment, including the evolution of resistance [119]. A priori, it is hard to predict how two treatments interact without insights into
the way cellular states change in response to one treatment, the average lifetime of such state changes and their relative sensitivity to subsequent perturbations. Such insights are critical both to optimize therapeutic regimes and to predict the effect of intra- and intertumor heterogeneity on the response to therapy.

### 3.1 Ionizing Radiation Leads to the Phenotypic Diversification of Isogenic Populations of Human Cells

DNA damage-based cancer therapy has been widely used in the clinic to treat a vast diversity of cancers, both as standalone therapy [120] or in combination with other agents [121]. However, the long-term of heterogeneity in cellular states in response to DNA damage and the way this initial perturbation modifies sensitivity to secondary treatments are not well understood. We used fluorescent live cell imaging to quantify the number and timing of division events that individual human cells experience in the course of 5 days after x-ray. We observed an irradiation dose dependent decrease in the total number of divisions that cells go through (Fig. 3.1A). Among dividing cells, there was substantial heterogeneity in the duration of the initial phase of cell cycle arrest, which lengthened on average with increasing irradiation dose (Fig. 3.1B). Variability in cell cycle arrest duration was higher than the variability in cell cycle length of unirradiated cycling cells (Fig. 3.1B). Therefore, phenotypic diversification emerges as a byproduct of exposure of isogenic human cells to DNA damage. We aimed to understand the consequence of such diversity in the context of combination cancer therapy.
3.2 Qualitative and quantitative re-shaping of DNA damage dose response curve in the context of combination treatment

Uncontrolled proliferation is a hallmark of human cancers [122] and therapeutic agents have been designed to selectively target proliferating cells at distinct stages of the cell cycle [121]. An instance of this therapeutic strategy is the inhibition of Eg5 kinesin, a molecular motor that is essential for mitosis [123]. Treatment of cells with the the selective Eg5 kinesin inhibitor S-Trityl-L-Cysteine (STLC) [124] triggers a prolonged mitosis arrest that can eventually lead to mitotic catastrophe and apoptosis or senescence [125, 126] (Fig. 3.2). To understand the way DNA damage interacts with Eg5 kinesin inhibition, we used empirical distributions of cell cycle arrest duration and division numbers (Fig. 3.1A, B) to simulate the outcome of combined treatment of irradiation and STLC (Fig. 3.3A). We predicted that DNA damage protects cells against the action of STLC through induction of transient cell cycle arrest, giving place to a non-monotonic dose response curve, where an intermediate dose of irradiation...
maximized cell proliferation (Fig. 3.3B). The shape of this curve can be understood as the result of a tradeoff between the protective effect of DNA damage and the cost it incurs in the form of reduced recovery potential (Fig. 3.3C). Interestingly, increasing duration of STLC exposure shifted the optimal damage dose to higher levels (Fig. 3.3B), suggesting that cells that arrest for longer, which have a strong disadvantage in the context of ionizing radiation treatment alone, gain a strong advantage in the context of chronic STLC exposure relative to non-irradiated cells.

To experimentally test this prediction, we treated cells with increasing doses of irradiation in the presence or absence of STLC for variable time durations (ranging from 1 to 3 days), and evaluated colony formation potential after STLC washout (Fig. 3.4). Consistent with our simulation, the combined treatment re-shaped the irradiation dose response curve from a monotonic decreasing to a non-monotonic increasing functional form (Fig. 3.4), where an intermediate dose of damage optimized long-term population growth. Moreover, increasing duration of STLC treatment led to a shift in the radiation dose that optimized cell proliferation. Taken together, our data shows that while the x-ray dose response curve changes qualitatively when DNA damage is combined with STLC treatment, it changes quantitatively as a function of STLC treatment schedule.
Figure 3.3: Simulations suggest re-shaping of DNA damage dose response in the context of combined treatment with STLC. **A.** We consider a situation in which cells are exposed to increasing doses of x-ray, in the presence or absence of STLC exposure, the duration of which can vary. Using individual cell division profiles, we estimate final population size by removing the progeny of cells that underwent mitosis while being exposed to STLC. **B.** Increasing duration of STLC decreases overall population growth. As compared to DNA damage alone, in which the final population size decreases with increasing dose of x-ray, treatment with STLC re-shapes this dose response to a non-monotonic increasing form. Increase in STLC duration leads to a shift in optimal dose of damage. Bold lines and shaded areas represent median and interquartile ranges of estimated population size of 30 bootstrap samples per condition. **C.** Re-shaping of dose response curve results from a trade-off between loss of proliferation potential as a result of DNA damage and the protective effect of cell cycle arrest in the context of STLC treatment.
Figure 3.4: Optimal dose of damage changes with STLC treatment schedule. Cells were treated with increasing doses of DNA damage in the presence or absence of STLC, with variable duration. Following STLC washout, cells were left to grow and fixed 11 days post-DNA damage. Nuclear staining allows estimation of population growth that results from treatment. Experimental results show non-monotonicity in the cellular response to DNA damage in the context of combined treatment with STLC. Moreover, optimal dose of damage changes as a function of duration of STLC exposure. Blue and orange arrows reflect directionality of selection due to the protective effect of DNA damage and loss of recovery potential, respectively.
Figure 3.5: Strong perturbation of p53 signaling abrogates non-monotonic interaction between DNA damage and STLC. A. The tumor suppressor protein p53 triggers cell cycle arrest in response to DNA damage. p53 is targeted to proteosomal degradation by its direct transcriptional target MDM2. B. The small molecule nutlin-3a inhibits the interaction between p53 and MDM2, resulting in p53 stabilization and cell cycle arrest potentiation. C. Homozygous knockout of p53 impairs cell cycle arrest upon DNA damage. D-G. Cells were treated with x-ray alone (D) or in combination with STLC for a period of 48h. Crystal violet staining was conducted 11 days post-irradiation to assess colony formation. Dose response to DNA damage was compared between cells with unperturbed p53 signaling (E), nutlin-3a mediated p53 stabilization (F), or in a p53^{-/-} background (G). Strong potentiation or abrogation of p53 signaling reshaped dose response from a non-monotonic to a monotonic decreasing functional form.
3.3 **Perturbation of p53 signaling re-shapes the response to combined radiation and STLC treatments**

We next investigated the molecular mechanism underlying the interaction between ionizing radiation and STLC. The tumor suppressor protein p53 is a master transcriptional regulator of the response of human cells to DNA damage and a critical mediator of the establishment and maintenance of cell cycle arrest [37, 127] (Fig. 3.5A). Perturbation of p53 signaling has strong consequences in response of human cells to DNA damage: p53 knockout cells fail to establish an arrested state and progress through the cell cycle in the presence of DNA damage [128] (Fig. 3.5B); conversely, pharmacological disruption of the interaction between p53 and its downstream negative regulator, the E3 ubiquitin ligase MDM2, leads to p53 stabilization and enforcement of the arrested state even in the absence of DNA damage [129] (Fig. 3.5C). To test whether p53 signaling modulates the interaction between irradiation and STLC treatments, we repeated the combined treatment of x-ray and STLC (48h schedule) in a p53--/ background. p53 knockout abrogated the interaction between x-ray and STLC (Fig. 3.5E, F), presumably due to impaired cell cycle arrest. In addition, p53 knockout conferred resistance to STLC across a wide range of doses (Fig. 3.5F), strengthening the notion that p53 is a critical mediator of the response to both ionizing radiation and STLC. To potentiate p53 signaling, we used the small molecule inhibitor nutlin-3a to stabilize p53 after damage, matching the duration of STLC exposure. We observed that nutlin-3a reshaped the dose response from a bell-shaped to a monotonic decreasing functional form (Fig. 3.5F). Since nutlin-3a is sufficient to induce cell cycle arrest in the absence of DNA damage, it is protective in the context of STLC treatment without incurring a cost associated with residual breaks. We concluded that either complete
abrogation or strong potentiation of p53 signaling eliminate the non-monotonic interaction between irradiation and STLC treatments.

### 3.4 Towards the Quantitative Modulation of Cell Cycle Arrest Duration

We are working towards understanding the way quantitative modulation of DNA damage signaling could affect cell cycle arrest duration and interaction with STLC. In response to double-stranded DNA breaks, sensory kinases such as ATM trigger p53 activation \[49, 50\], which leads to the production of the Cyclin-dependent kinase (CDK) inhibitor p21 \[53\], a direct effector of cell cycle arrest \[18, 19, 106\] (Fig. 3.6A). Previous work revealed that quantitative variability in p21 levels relates to heterogeneity in cell fate (Chapter 2). We used a cell line harboring a fluorescent fusion allele of p21 (Fig. 3.5B) and tracked the dynamics of this protein in the course of 5 days post-irradiation (Fig. 3.5C). We found that levels of p21 induction correlated with the duration of transient arrest across DNA damage doses (Fig. 3.5D). Using small molecule inhibitors for kinases upstream of p53, we are testing whether quantitative modulation of the p53-p21 signaling axis can change properties of arrest-exit time distributions, including the average and the variance. It will be interesting to explore the way quantitative changes in input-output relationships within DNA damage signaling can change conditions of optimality in the context of combined irradiation and STLC treatment.

Surveys of phenotypic states at the single cell level have highlighted a role for non-genetic heterogeneity in allowing specific systems to cope with changes in their
Figure 3.6: Levels of p21 induction correlate with cell cycle arrest duration after DNA damage. A. The CDK inhibitor p21 is a major effector of cell cycle arrest in response to DNA damage. B. An endogenously tagged p21 protein allows quantification of p21 levels in individual cells. C. Representative trajectories of p21 induction in individual cells after DNA damage. Dashed line denotes timing of first G1-S transition post-damage, as quantified by sharp degradation of p21 (Chapter 2). Solid lines denote mitosis events. D. Average levels of p21 induction correlate with timing of cell cycle re-entry in arrested cells, across doses of x-ray.
environment [130, 131]. In particular, dormant states have been linked with fractional killing in the context of antibiotic treatment [77] and chemotherapy [66, 75]; while non-genetic heterogeneity can arise spontaneously in populations due to intrinsic stochasticity in biochemical systems [28, 63], concerted phenotypic diversification of cellular populations can result as a consequence of heterogeneity in cellular signaling in response to acute perturbations. Here we show that diversity in the establishment and duration of transient cell cycle arrest in response to DNA damage can re-shape the cellular response to the mitosis-targeting agent STLC. Antagonism between DNA damage and STLC generates a tradeoff within which an intermediate dose of damage optimizes cell viability. Such optimal damage dose depends critically on STLC treatment schedule. Recent work has highlighted the way timing and duration of treatments in the context of combination therapy can shape the response of cancer cells to treatment [132–135]. It will be important to elucidate the extent to which cells can modulate cellular signaling to match perturbation timescales in their path to resistance to dynamic therapeutic strategies.

3.5 Materials and Methods

Materials

Cell Culture

RPE cells immortalized with telomerase overexpression (RPE-hTERT, a kind gift from S.J. Elledge, Harvard Medical School) were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, 100mg/mL streptomycin and 250ng/mL fungizone (Gemini Bio-Products). For microscopy, DMEM/F12 + 5% FBS lacking phenol red and riboflavin, was used. When necessary, media was
supplemented with selective antibiotics (400 μg/mL G418, 2 μg/mL puromycin).

To endogenously tag p21 with the fluorescent protein mKate2, we used the eFlut toolset as previously described (Stewart-Ornstein, 2016). The resulting tagged coding sequence includes a P2A cleavable Neomycin resistance protein, allowing selection for cells harboring the tag without disrupting the endogenous p21 3'UTR. Single cell clones were expanded through limited dilution and subsequently screened for marker expression. Cells were further infected with iRFP-NLS lentiviral vector, a constitutive nuclear marker.

**Irradiation**

Cells were exposed to time-controlled doses of x-ray.

**Reagents**

Crystal violet (cat #C0775-25G) and Glutaraldehyde solution (cat #G6257-100ML) were purchased from Sigma. Nutlin3-a (Sigma cat #SML0580-5MG) and S-Trityl-L-Cysteine (Sigma cat #164739-5G) were stored at -20°C in 5 mg/mL and 50 μM DMSO stocks, respectively. DAPI (cat #D21490) was purchased from Life Technologies.

**Methods**

**Live-Cell Microscopy**

Cells were grown in poly-D-lysine-coated glass bottom plates (MatTek Corporation) and imaged using a Nikon Eclipse TE-2000 inverted microscope with a 10X Plan Apo objective and a Hamamatsu Orca ER camera, equipped with environmental chamber controlling temperature, atmosphere (5% CO2) and humidity. For long-term live cell imaging experiments (>5 days), media was replaced every day to maintain growth.
factor stimulation throughout the entire duration of imaging. Images were acquired every 30 min using the MetaMorph Software.

**Single Cell Tracking and quantification**

A semi-automated method was developed that allows tracking and cell fate annotation of individual, fast moving cells over long timescales. Our method relies on (i) automatic identification of single cell centroids using intensity and shape information of a constitutive nuclear marker; (ii) centroid linkage and track propagation using nearest-neighbor criteria; and (iii) real-time user correction of tracking, and annotation of cell fate events. Tracking data was then used to quantify intensity of fluorescent reporters from background subtracted images by averaging 10 pixels within the cell nucleus. Matlab scripts for tracking and quantification of single cell data are available upon request.

**Simulation of combined radiation and STLC treatment**

To simulate the effect of combined treatment of x-ray and STLC on the overall cell population growth, single cell division profiles obtained through live-cell imaging were used to estimate the expected progeny size of each cell present at the beginning of the experiment; such estimate assumed homogeneity in cell fate (number and timing of division events) among sister cells. To incorporate the effect of STLC treatment, it was assumed that cells that divided within the specified duration of STLC exposure lost potential to divide, and therefore did not contribute to the overall growth of the population. To account for undersampling and cell-to-cell heterogeneity in fate, the effect of each treatment schedule was estimated in 30 independent bootstrap samples obtained through sampling with replacement the original set of progenitor cells.
Colony formation assay

Cells were plated in 6 well plastic dishes (Corning) and were exposed to x-ray irradiation one day after. Cells were treated with STLC and/or Nutlin3-a after irradiation. Individual wells were washed two times with PBS at specified schedules (main text) and replaced with fresh media. Cells were fixed 9–11 days post-irradiation, depending on treatment schedule, when discrete and well-separated colonies became visible. Recovery was assessed through DAPI nuclear staining or Crystal violet staining.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature at the appropriate time after treatment. Cells were permeabilized with 0.1% Triton-X and blocked with 2% BSA in 1X PBS. Cells were incubated with DAPI, followed by a three PBS washes. Wells were scanned using a Nikon Eclipse TE-2000 inverted microscope with a 2X Plan Apo objective and a Hammamatsu Orca ER camera.

Crystal violet staining

Crystal violet staining was conducted as described in [136]. Briefly, wells were washed with PBS and fixed with glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) solution for 30 min. After removing crystal violet, wells were submerged in water to remove excess solution and were dried overnight at room temperature. Plates were imaged with a flatbed scanner (Hewlett-Packard).
3.6 **Acknowledgements**

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3.7 **Author Contributions**

José Reyes conducted experiments and analyzed data; José Reyes and Galit Lahav wrote manuscript.
A continuum model of cell fate in the response of human cells to ionizing radiation

The discreteness of cellular states can be blurred out by population averaging. Such a picture is oftentimes invoked to justify the need of single cell resolution in the quantification of molecular and phenotypic traits to accurately represent the diversity of states within a cell population. Our investigations of the long-term fate of human cells after exposure to ionizing radiation resulted in the reverse picture: while a static characterization of cellular states after DNA damage would reveal two discrete cell populations corresponding to cycling and arrested states, the discreteness of these two states is blurred out when taking into consideration whole histories of individual cell behavior. As cells switch in and out of the arrested state with
heterogeneous rates over long timescales, it is possible to trace a phenotypic continuum connecting cells that divide periodically and those that don’t divide in the course of one week after DNA damage.

Cellular senescence is regarded as an irreversible form of cell cycle arrest that stands in contrast with the reversible arrested state known as quiescence. The cell fate continuum revealed by single cell division profiling makes it difficult, if not impossible, to establish a hard boundary between quiescent and senescent phenotypes. In fact, from the perspective of G1 arrest alone, senescent cells don’t behave in a way that is fundamentally distinct from that of quiescent cells. Rather, arrested cells represent a mixture of cellular phenotypes with variable resilience against perturbations of environmental or intracellular origin. By this we don’t suggest that senescent and quiescent cells are equivalent in terms of cell physiology. Consistent with long-standing observations in the field [88, 94], damaged cells lose recovery potential and escape events don’t result in the resumption of processive proliferation. Moreover, senescent cells are known to activate specific gene expressions programs, such as the senescence-associated secretory phenotype [137]. The phenotypic plasticity of senescent cells and the role of non-genetic heterogeneity in DNA damage signal processing that we report here add a new dimension to the study of the functional consequences of senescent cells in the context of tissue homeostasis during organismal ageing [1, 138, 139], tumorigenesis [97, 140] and the response of cancer cells to cytotoxic chemotherapy [99].

Our intuition of cell fate after ionizing radiation changed dramatically depending on the duration of our observations. A large fraction of cells that seemed to be stably arrested during the first few days of the response eventually escaped the arrested state and divided when we tracked them for an entire week. Following the same reasoning,
Figure 4.1: Month-long timecourse suggests occurrence of escape events beyond one week after DNA damage. 

A. Lack of colony formation after 20Gy γ-irradiation, as evidenced by crystal violet staining, allows longitudinal observations of irradiated samples (weeks) without the confounding effect generated by a small subpopulation of actively proliferating cells. 

B. Schematic of experimental strategy. Cells were exposed to 20Gy γ-irradiation and maintained in IdU supplemented media for 27 days, changing media every 4 days. IdU incorporation allows keeping track of cells that went through at least one round of replication without the need to follow individual cells over time. IdU was added after the first day post-damage to avoid incorporation in cells that were in S-phase at the time of irradiation. 

C. Single snapshots of irradiated samples show negligible frequency of proliferating cells throughout the month, as evidenced by the proliferation marker Ki67. 

D. Frequency of IdU positive cells increases slowly throughout the timecourse, suggesting that most cells escape arrest at least once in the course of one month after irradiation.
it is tempting to speculate that a large fraction of cells that did not divide during this first week could eventually escape, a phenomenon that would be detectable only by increasing the duration of our observations. Our ability to follow individual cells becomes increasingly limited over time due to the effects of phototoxicity and stress associated with the microscopy environment. To gain insights into the stability of the arrested state beyond one week after damage, we exposed cells to 20 Gy γ-irradiation, a dose under which there is no long-term colony formation (Fig. 4.1A), and maintained cells with IdU supplemented media (Fig. 4.1B). Incorporation of IdU to genomic DNA allowed us to identify cells that went through at least one round of replication up until the time of sampling. In a month-long timecourse, irradiated cells appeared uniformly arrested, as quantified by the proliferation marker Ki67 (Fig. 4.1C), but showed progressive accumulation of IdU. At the last time point (day 27), most of the cells present in the plate had gone through S-phase at least once after DNA damage (Fig. 4.1D). While the fraction of IdU positive cells is hard to interpret without knowing the distribution of the number of cell divisions during this whole month, our data suggests that escape events keep happening beyond one week after damage.

4.1 HETEROGENEITY IN CELL FATE AFTER IONIZING RADIATION IS PRESENT ALONG TWO AXES: CELL CYCLE ARREST DURATION AND RECOVERY POTENTIAL

We observed substantial heterogeneity in the duration of cell cycle arrest, ranging from a few hours in fast recovering cells to several days in cells that we call escapers; as noted above, such heterogeneity could potentially extend up to one month post-DNA damage. In light of our observations, cell fate in response to ionizing radiation may
not be a matter of whether a cell will escape G1 arrest or not, but how long damaged cells are able to maintain the arrested state for. It is thus critical to understand the mechanistic basis underlying heterogeneity in the timing of arrest exit and the relative contribution of the initial state of the cell, the number of double-stranded DNA breaks it received, and the amount of damage that persist over long-timescales to the overall heterogeneity in escape timing. It will be important to study the way the distribution and dynamics of signal transducers such as p53 shape the distribution of escape timing. Heterogeneity in cell cycle arrest duration can have profound consequences in the context of combination cancer therapy (Chapter 3). A quantitative account of the way this temporal dimension of cell fate is regulated will be informative to predict potential adaptive trajectories of cancer cells when treated with dynamic therapeutic schedules, and will provide the ground to reshape exit time distributions through pharmacological perturbations.

Heterogeneity in cell fate after ionizing radiation is not manifested only in terms of cell cycle arrest duration, but also in terms of long-term recovery potential. This is particularly important in the context of resistance to DNA damage based therapies and early tumorigenesis, as it is the determinant of the capacity of cancer cells to form and maintain a tumor. Single cell division profiles revealed an inverse correlation between recovery potential and duration of cell cycle arrest: a small proportion of damaged cells recover early and divide in a pattern that is indistinguishable from non-irradiated cells, contributing disproportionately to the final cell population due to exponential growth in tissue culture; on the other hand, cells that divide late have a tendency to either re-enforce the arrested state after a single division or go through subsequent rounds of cell division with an extended cell cycle duration. DNA damage could potentially account for this inverse correlation. Although it has not been conclusively shown at
the single cell level, the amount of damage is expected to both increase cell cycle ar-
rest duration and decrease colony formation potential (i.e. by increasing chromosome
missegregation rates). However, this does not necessarily imply that these two di-
mensions of cell fate are intrinsically coupled. On the contrary, we have shown that
stabilization of p53 using the small molecule nutlin-3a can trigger a prolonged arrest
without complete loss of colony formation potential. It is possible that different trans-
formed and non-transformed human cell lines could inhabit distinct parts of the space
defined by these two cell fate axes (cell cycle arrest duration and recovery potential).
Future work is bound to understand the consequences of such diversity in the context
of radiation treatment, either as single agent or in combination.

4.2 Unexplained variability in the vicinity of arrest escape
events

The quantification of DNA damage signaling at different levels of the biochemical cas-
cade linking damage sensing with cell cycle arrest allowed the identification of one con-
dition under which homeostasis of the G1 arrested state is broken. Double-negative
feedback between p21 and CylinE-CDK2 can either buffer or amplify fluctuations in
p21 levels that stem from upstream p53 signaling, depending on the average level of
p53 and p21 that the system is operating in. However, not every escape event can be
explained by a transient reduction of p53 pulse amplitude in the vicinity of the tran-
sition. It is possible that p53 trajectories hold more information than what we have
been able to extract with our current data analysis framework. A particularly exciting
direction is the consideration of trajectories themselves as random variables, comple-
menting the timepoint-by-timepoint analysis that we report in this work. The space
of all possible trajectories is dauntingly large, and this research direction will require an increase in the throughput of protein dynamics and cell fate quantification in live single cells, as well as the development theoretical tools aimed at representing and manipulating distributions of cellular histories.

p53 signaling coexists with other signaling axes that collectively shape the response of human cells to ionizing radiation [36]. While some signaling pathways act in parallel with p53 to enforce cell cycle arrest after DNA damage, other pathways antagonize DNA damage signaling to promote cell cycle re-entry. Through the quantification of p53 levels and dynamics in individual cells as they entered and exited cell cycle arrest, we learned that fluctuations in p53 levels are subject to amplification by the downstream p21/CDK2 circuit and contribute to escape from the arrested state. Similar quantitative arguments can be made when considering other signaling pathways that converge on the modulation of p21 levels and CDK2 activity. For instance, growth factor signaling through ERK plays a major role in the regulation of mammalian cell proliferation [58] and thus heterogeneity in ERK activity could also contribute to escape from cell cycle arrest. As our ability to survey the dynamics of multiple molecular players that carry information within the same cell grows, it will be possible to understand the extent to which interference between distinct dynamical signals comes into play to determine whether a cell remains arrested or re-enters the cell cycle.

4.3 Concluding remarks

By quantifying the full history of cell fate in individual cells exposed to ionizing radiation we revealed a continuum of cellular outcomes, which stand in contrast to the discreteness and apparent stability of cellular states observable in single snapshots of
irradiated cell populations. To a first approximation, this phenotypic continuum can be described in terms of cell cycle arrest duration and recovery potential. Using live cell imaging to quantify the levels and dynamics of p53 and p21 proteins, which link DNA damage sensing and cell cycle arrest, we revealed that heterogeneity in these two key molecular players contribute to escape from cell cycle arrest in two different ways: (i) the average levels of p53 and p21 induction correlate with the overall probability of escape; and (ii) a transient decrease of p53 protein levels precedes the onset of actual escape events. Since escape from arrest is frequently associated with chromosome missegregation, we speculate that noise-driven destabilization of cell cycle arrest can potentially precede and contribute to genetic changes associated with the early onset of tumorigenesis and resistance to cytotoxic chemotherapy.

Our work revealed substantial heterogeneity in the duration of cell cycle arrest, ranging from a few hours to several days and potentially weeks. Future work is bound to understand whether these widely distinct timescales can be mechanistically unified in terms escape from cell cycle arrest at the edge of G1-S transitions. The quantification of signaling dynamics in individual cells should provide a framework to understand how cells regulate this temporal dimension of fate after DNA damage, and the suggest experimental interventions aimed at quantitatively modulating arrest-exit time distributions. Such an understanding is critical for the design of optimal dynamic therapeutic strategies and for predicting the potential trajectories that cancer cells could follow in their path towards resistance to such treatment schedules.
Appendices
p53Cinema allows tracking and quantification of individual cell lineages

Our ability to query the dynamics of single cell responses to stimuli critically depends on our capacity to follow individual cells. Phenotypic and molecular heterogeneity can unfold over diverse timescales, that range from minutes and hours in the case of the response to acute perturbations [57–60], to fractions of a day in the case of circadian oscillations and cell cycle transitions [104, 141, 142] and days in the case of cell fate decisions such as cell cycle arrest, cell death and differentiation [75, 143]. A comprehensive account of the dynamical character of signal processing within cells requires us to reliably gather individual cell trajectories encompassing the full history of the cellular response to specific perturbations. More-
over, collection of large numbers of single cell trajectories is paramount for analytical strategies that leverage cell-to-cell heterogeneity to relate quantitative changes in signaling to specific cellular outcomes.

A.1 **SEVERAL FACTORS LIMIT THE RELIABLE IMPLEMENTATION OF AUTOMATIC TRACKING OF INDIVIDUAL CELLS ACROSS MULTIPLE GENERATIONS**

Several factors limit our ability to reliably collect continuous single cell trajectories in the course of several days. Cell motility imposes unique challenges to this task: only a fraction of cells present initially in the field of view remain within it for the entire duration of the experiment, making it necessary to collect and process a larger number of fields of view when working with fast-moving cells as compared to slower cell lines. Even when cells remain in the field of view, constant cell-cell interactions can confuse automatic algorithms designed to identify individual cells. Cell-cell interactions can also affect the speed and direction of movement, and a common tracking error occurs when algorithms propagate tracks along the incorrect cell path when two cells transiently interact.

Over long timescales (several days), basic cellular features such as size and morphology can dramatically change. In fast dividing populations, even a sparsely plated dish can become confluent within a few days. Confluent cells will tend to appear elongated and squished. On the other hand, specific perturbations such as DNA damage can induce cell cycle arrest, during which cells continue to grow in size without cell division. Image processing algorithms need to be able to adapt to these changes, and the static parametrization based on cellular features present at the beginning of the experiment...
can become unreliable at a future time point.

Computer vision and image processing are fast growing fields, and multiple computational strategies have been developed to segment and track individual cells [144–147]. A general scheme of tracking relies on two basic steps: first, individual cells are identified and segmented of cells using fluorescent markers, such as nuclear localized fluorescent proteins or fluorescently tagged histones; and second, cells are linked through sequential frames to assemble full single cell tracks. This second step relies on specific criteria to accept or reject potential linkages. The simplest of these criteria is nearest neighbor matching, where a cell is linked to the closest cell in the next frame. More sophisticated information can be used to increase linkage accuracy, such as incorporation of local motion patterns (such as direction, velocity and acceleration) to predict where a particular cell would be in the next time point. A global optimization strategy has been used to find the most parsimonious linkage between multiple cells in one frame to the next [147]. Furthermore, incorporation of information about shape and levels of molecular markers can increase accuracy of linkage, if an appropriate background model of temporal variation of such markers is available. More recently, joint optimization of segmentation and tracking provides a promising direction to improve these two image analysis tasks [145].

It is often suggested that increasing the frequency of imaging can greatly enhance track accuracy. A rule of thumb is that the frequency of imaging has to be such that, on average, a cell doesn’t move more than one cell diameter between time points. Often times, this is not possible due to the velocity at which cells move, the intrinsic limitations in acquisition speed and increased phototoxicity associated with periodic fluorescent illumination. Errors in tracking are amplified in the context of timelapse data, as compared to static analysis of single cell populations: a single tracking error can
make a whole track unusable and it is not necessarily straightforward to automatically detect and exclude errors with tracks. It is important to note that many of the challenges faced by tracking algorithms are intrinsic properties of experimental systems; while some of these can be overcome by the use of more sophisticated experimental strategies, such as the use of micropatterning to maintain fast moving cells within a field of view for extended time durations, the image analysis community should aim to improve computational strategies to match the ability of human subjects to visually track fast moving and interacting objects.

A.2 **P53Cinema allows collection of high confidence single cell tracking data**

The overarching goal of the development of single cell tracking tools is to achieve accurate and automatic single cell tracking and segmentation over long timescales. Critically, such methods should also be able to detect critical cell fate events such as cell division and cell death. While the field is moving forward in this direction and automatic algorithms are bound to reach a level of accuracy suitable for experimental inquiry, our approach here was to develop a tracking system that would allow researchers to collect a large number of high confidence single cell trajectories through the combination of semi-automatic tracking and manual user curation. Our tracking system relies on: (i) identification of single cell centroids using intensity or shape information from fluorescent markers, (ii) linkage of cells in sequential frames and automatic track propagation, (iii) user guided track curation when there is ambiguity in linkage, as well as annotation of cell fate events (Fig. A.1).
Identification of individual cell centroids (shape and intensity information)
Sequential linkage of centroids from frame to frame (nearest neighbor criterion)
Single cell track propagation

Manual identification of missing centroids
Real-time linkage correction
Resolution of ambiguity points
Cell fate annotation

Table of linked centroids per image sequence
Custom single cell quantification and analysis scripts

Figure A.1: p53Cinema tracking strategy at a glance. p53Cinema combines automatic centroid identification and track propagation with user curation and correction for the collection of high-confidence single cell tracking data. The current implementation is general enough to accommodate more sophisticated strategies to identify and link centroids through sequential frames. The output of our tracking system can be used together with the single cell quantification and analysis software included in the p53Cinema package, or it can be used as an input to custom scripts through the intersection of centroid coordinates.
The current implementation of the p53Cinema tracking software identifies single cell centroids as local maxima in blurred intensity or distance transformed images (shape criterion). Furthermore, it uses a reciprocal nearest neighbor criterion to extend single cell tracks from frame to frame. A single cell can be tracked by selecting a centroid in a frame and scrolling forwards or backwards to extend tracks in real-time. Alternatively, users can choose to automatically propagate tracks and bypass the need to manually explore the image sequence. The tracking engine automatically stops when it fails to find a centroid within range or when it cannot decide between two competing candidate centroids to link the track into. Users can aid tracking on-the-fly by (i) introducing centroids that the automatic algorithm failed to identify (most commonly due to heterogeneity in intensity levels of fluorescent markers), (ii) backtracking and correcting miss-tracked cells and (iii) resolving ambiguity in automatic track propagation by selecting the correct centroid among competing candidates. Moreover, users can manually annotate cell fate events, such as cell division and cell death, and link sister cells for the purpose of within lineage analysis. Our tracking system is general enough to accommodate potential extensions, such as the incorporation of pre-processed centroid information from more sophisticated algorithms such as active contour based segmentation, a Kalman filter implementation to improve linkage accuracy based on the pattern of cell movement, and the use of neighbor cell tracks to resolve potential ambiguity.

The output of p53Cinema is a tab delimited table containing the position of centroids in individual frames and timepoints; such coordinates are linked with a unique cell identifier that can be effectively used to reconstruct individual cell tracks. We provide tools to quantify the signal of fluorescent reporters and extract cell fate information from tracking data for downstream analyses. In addition, it is possible to inter-
sect the output coordinates from our software to merge p53Cinema based tracking with custom segmentation and quantification scripts. We envision that our tracking system will be of value for researchers collecting and analyzing single cell data, for developers to test novel strategies to link cells from frame to frame, and for the generation of gold standard datasets needed by the image analysis community to benchmark new tracking methods.

### A.3 Single cell lineages reveal heritability of G1 duration

As a proof of principle, we quantified behavior of cycling human cells imaged in the course of three days with daily media replacement to ensure growth factor stimulation throughout the entire duration of the experiment. We used p53Cinema to track full individual cell lineages through multiple cell generations (Fig. A.2A, B). Using the mVenus-hGeminin(1-110) fluorescent reporter [104], we quantified cell cycle progression in along individual cell tracks (Fig. A.2C). We integrated genealogical relationships between cells with the duration of individual cell cycle phases within the lineage to understand whether variability in cell cycle duration is inherited from one cell generation to the next (Fig. A.2D).

Overall, we collected 829 non-redundant mother-siblings trios and asked whether long cell cycles in daughter cells are preceded by statistically longer cell cycles in mother cells. We focused our analysis on the heritability of G1 duration, which accounts for most of the variability in overall cell cycle length (Chapter 2). In general, for a given G1 duration threshold $t$, mother-sibling trios can be classified in three categories: (i) a situation in which the two sister cells have $G_1 > t$ (concordant,
Figure A.2: p53Cinema allows tracking of individual cell lineages across multiple generations. A. Individual proliferating human cells were imaged for three days every 30 min. Four fields of view were merged to maximize complete cell tracks. The progeny of cells present at the beginning of the experiment were tracked for as long as possible (the end of the image sequence, or when they cells left the field of view). Each track is represented as a gray line. The red box encloses cells corresponding to a particular lineage. Within this family, tracks are highlighted with colors corresponding to cell generation. Yellow circles represent division events. B. Zoom-in to box highlighted in (A) C. The mVenus-hGeminin fluorescent reporter accumulates at the onset of the G1-S transition and degrades sharply upon mitosis, allowing quantification of G1 and S/G2 duration. D. Single cell lineage highlighted in (A,B). mVenus-hGeminin allows quantification of G1 duration within specific branches of this family tree.
Figure A.3: Single cell lineages reveal heritability of G1 duration. A. Three general configurations of G1 duration distinguish mother-siblings trios. B. Daughter G1 duration was classified as lengthened or shortened on the basis of a threshold $t$. Average +/- s.e.m. G1 duration of mother cells is quantified as a function of the G1 length threshold $t$.

lengthened sisters), (ii) a situation in which only one sister has $G_1 > t$ (discordant sisters) and (iii) when the two sister cells have $G_1 < t$ (concordant, shortened sisters) (Fig. A.3A). We found that longer G1 duration in daughter cells was preceded by a longer than average G1 duration in mothers (Fig. A.3B). This effect was more pronounced in concordant cells with lengthened G1 duration than in discordant cells (Fig. A.3B, purple vs pink plots). Our statistical analysis of variability in G1 duration along single cell lineages revealed a signature of heritability in cell cycle length. It will be important to understand the mechanistic basis of lengthened G1 duration inheritance and the number of cell generations this memory lasts for.

A.4  SOFTWARE INFORMATION

The p53Cinema tracking package is freely available to the research community under the GNU license. (https://github.com/balvahal/p53CinemaManual/releases).
System requirements: Matlab 2013b or higher.

Input data: Individual grayscale images in TIF format. The user should also provide a tab delimited file containing the following columns: filename[STRING], group_label[STRING], channel_name[STRING], position_number[NUMERIC] and timepoint[NUMERIC].

A.5 Author contributions

José Reyes and Kyle W. Karhohs conducted experiments and developed image analysis framework; José Reyes analyzed data; Jia-Yun Chen established the mVenus-hGeminin(1-110) RPE cell line.
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76


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