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Dynamics of p53 in response to DNA damage vary across cell lines and are shaped by efficiency of DNA repair and activity of the kinase ATM

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Abstract

Cellular systems show a wide range of signaling dynamics. Many of these dynamics are highly stereotyped, such as oscillations at a fixed frequency. However, most studies looking at the role of signaling dynamics focus on one or a few cell lines, leaving the diversity of dynamics across tissues or cell lines a largely unexplored question. Here we focused on the dynamics of the tumor suppressor protein p53, which in response to ionizing radiation was shown to exhibit oscillations that play a role in the response to DNA damage. We established live cell reporters for 12 cancer cell lines expressing wild-type p53 and quantified p53 dynamics in response to a range of double strand break inducing DNA damage doses. In many of the tested cell lines, we found that p53 oscillates and the periodicity of the oscillations was fixed. Other cell lines exhibited distinct dynamic behaviors, including a single broad pulse or a continuous induction. By combining single cell assays of p53 signaling dynamics, small molecule screening approaches in live-cells, and mathematical modeling, we identified molecules that perturb p53 dynamics and determined that cell-specific variation in the efficiency of DNA repair and the activity of the kinase ATM controlled the signaling landscape defining p53 dynamics. Because the dynamics of wild-type p53 varied substantially between cell lines, our study highlights the limitation of using one line as a model system and emphasizes the importance of studying the dynamics of other critical signaling pathways across different cell lines and genetic backgrounds.

Introduction

Many signaling pathways employ complex dynamics to encode information about intensity, duration, and identity of a signal. The mechanism and differential outcomes of this encoding have received substantial attention, but less emphasis has been put on the conservation of these dynamics across different contexts or cell types. For example, pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), nuclear factor of activated T cells (NFAT), and extracellular signal-regulated kinase (ERK) all show complex time dynamics in mammalian cells after stimulus, but rarely has the diversity of these

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dynamics across tissues or cell lines been explored^{1–3}. The conservation of dynamical behaviors across cell lines encodes important information about the genetic or epigenetic underpinnings of these responses.

The dynamics of signaling pathways are increasingly seen as potential clinical targets for cancer therapy⁴. Understanding the diversity and dose dependence of these dynamics is therefore crucial to predict potential toxicities in the body and which tumors may be sensitive to certain timescales of treatments. In addition, choosing appropriate model systems or cell lines to represent a relevant clinical spectrum of behavior is a challenging unsolved problem in basic research. Understanding the robustness of a dynamic behavior across cell types or cancer lines is therefore required for developing greater mechanistic insights into the conservation or dynamic range of specific features of various cellular systems.

Previous work on the response of the tumor suppressing transcription factor p53 to DNA damage suggests that p53 signaling has dynamic properties that depend on the stimulus and can alter the outcome of DNA damage. In response to double strand breaks, feedback loops cause p53 to oscillate in populations and individual cells^{5,6}, a pattern of signaling compatible with both resumption of proliferation or permanent arrest if such oscillations persist. In contrast, non-oscillatory sustained activation of p53 is associated with permanent cell cycle arrest⁷. Although oscillatory expression of p53 has been observed in several cell types^{8,9} and in vivo¹⁰, it is unclear if this represents a universal signaling pattern or a special case, and further, how these dynamics might play out in cancer cells with a compromised DNA damage response.

To explore the diversity in p53 signaling, we collected a set of twelve p53 wild-type tumor cell lines and quantified the response of the p53 protein to DNA damage in individual cells. We found that all twelve lines respond to DNA damage by activating a functional p53. However, the dynamics of p53 varied greatly across cell lines. Further, in some cell lines the p53 response was largely dose independent, whereas other lines showed dose responsive behaviors. To identify what cellular features might lead to different p53 dynamics, we applied a targeted chemical screen for modifiers of p53 dynamics and determined DNA repair capacity and ATM signaling as the main dynamical regulators. Small molecule inhibitors of ATM converted sustained p53 signaling to the classical pulsatile behavior, whereas inhibitors of DNA repair, or compounds which increase ATM activity, promoted sustained signaling. We codified our experimental findings in a mathematical framework which describes the “signaling space” of p53 across cell lines and DNA damage doses. Our study reveals that wild-type p53 shows different dynamical behaviors across cell lines, which are shaped by specific signaling states and suggests pharmacological approaches to modulate these dynamics.

Results

Different Cell lines show comparable absolute p53 abundance but diverse p53 dynamics

To study the diversity in p53 response to DNA damage, we assembled twelve cell lines including 11 of the 14 NCI60 cell lines that are wild type for p53 (discarding three lines that

posed difficulties for microscopy) and the widely-used p53 wild type osteosarcoma cell line U2OS. We acknowledge that this is not a random selection of cell lines as it includes an overrepresentation of melanoma lines (5/12, due to the relatively low rate of p53 mutations in melanoma). However, these lines represent a fairly typical range of immortalized cell lines that are widely used in the cancer and DNA damage fields. The lines were treated with the radiomimetic drug neocarzinostatin (NCS), and p53 abundance was measured by immunofluorescence (IF). NCS creates a burst of double strand DNA breaks and was shown to act within 5 min following its addition to culture medium¹¹. All twelve lines showed comparable basal amounts of p53 (mean abundance within threefold), and after DNA damage, p53 abundance increased in every cell line from 1.25–5 fold (Fig. 1, A and B). Cell-to-cell variation was substantial in all lines, with damaged and undamaged populations overlapping in p53 abundance. This variation in p53 abundance is not likely the result of genetic inhomogeneity across a cell line, because multiple clones obtained from 2 lines (HCT116 and A549) showed similar degrees of heterogeneity and relatively uniform responses to DNA damage (fig. S1).

To test the dependence of p53 induction on the extent of DNA damage, we quantified p53 abundance in response to a titration series of NCS in each cell line. We applied NCS in doses ranging from those that completely inhibit growth to those that cause minimal loss of proliferation (two orders of magnitude of NCS; fig. S2A), and measured p53 abundance after 2 hours (early response) and eight hours (mid response). In the A549 line, for example, we observed an initial increase in p53, which was comparable across all DNA damage doses (measurement at 2 hours; Fig. 1C). In contrast, the mid response (at eight hours) was graded, with a linear correlation between damage dose and p53 abundance (Fig. 1D). A similar picture was obtained for the other lines (Fig. 1, E and F), although we note that three of the melanoma lines (UACC257, SK-MEL5 and MALME3) showed a slight dose dependency at the 2-hour time point. Together, these results suggest that p53 initial induction is switch-like while the later response is more dose-dependent. This is consistent with an “emergency” DNA damage signaling system that initially responds at maximum strength regardless of the insult strength, whereas the mid time-point reflects the contribution of DNA repair and transcriptional feedback loops regulating p53¹².

The major feedback mechanism that attenuates p53 is its transcriptional target gene encoding the E3-ligase MDM2^{5,13}. To quantify the p53-MDM2 feedback in all twelve lines, we measured the abundance of p53 and MDM2 at the early (2 hour) and mid (8 hour) response to DNA damage (NCS) by immunofluorescence. In the early response, we observed an increase in p53 and a minimal change in MDM2 across all cell lines (Fig. 2, A and B). In the mid response, all cell lines showed an increase in MDM2 abundance, suggesting that p53 transcriptional activity is intact (Fig. 2B and fig S2B). The abundance of cyclin-dependent kinase (CDK) inhibitor CDKN1A (also known as p21), encoded by a second p53-dependent gene, also increased after DNA damage in all cell lines (fig. S2C). At 8 hours, the change in p53 abundance varied substantially, with increases in some lines and losses in others (Fig. 2A). Time course measurements of p53 abundance in fixed MCF7, A549, and HCT116 cells, which were assayed at additional intermediate (5 hours) and late (24 hours) time-points, suggested different dynamical behaviors of p53 in these three lines (Fig. 2C): an oscillatory pattern in MCF7 cells; a broader wave of activation in A549 cells;

and a continuous accumulation in HCT116 (Fig. 2C). Analysis of these three representative cell lines suggests that p53 dynamics may vary considerably across cell lines.

Live cell measurement of p53 dynamics show cell-line specific behaviors

To further examine p53 dynamics across cell lines, we constructed live cell reporters by transducing an exogenous allele of p53 tagged with yellow fluorescent protein (p53-YFP) into each of the 12 cell lines. We selected stably expressing clones for each line (with the exception of LOX-IMVI, for which we were unable to obtain a single cell clone and used an enriched polyclonal population), and assayed p53 dynamics in response to DNA damage by live-cell microscopy (Fig. 3A). For these experiments, we used ionizing radiation (IR) to induce DNA damage because it offered better day-to-day reproducibility than NCS across a wide range of doses. MCF7 cells showed sustained pulses of p53 after exposure to 6Gy IR, and one pulse after exposure to 1Gy IR (Fig. 3B). In contrast, A549 cells showed more complex dose dependent dynamics, with cells pulsing regularly after 1Gy IR, but showing a broader p53 pulse after 6Gy (Fig. 3B). To examine the dependency of p53 dynamics on the radiation dose across cell lines, we exposed each line to 5 doses of IR (1, 2, 4, 6, and 8Gy) and imaged single cells for 24 hours. Individual cells were tracked over time for p53 abundance and cell division events. These doses of radiation were sufficient to markedly suppress cell growth (Fig. 3C). Specifically, all cell lines showed a large fraction of actively dividing cells after exposure to 1Gy IR, with that fraction declining as the dose of IR increased. Only a few cell lines showed notable division at 4Gy, and no divisions in any cell line were observed after exposure to 6Gy (Fig. 3C).

All cell lines showed induction of p53 in response to radiation. However, as suggested by our experiments in fixed cells (Fig. 2), its dynamics varied considerably between the cell lines. This variation was independent of the tissue of origin, as the five melanoma lines we studied showed a range of p53 dynamics. Our analysis revealed one major group of cell lines with periodic p53 pulses at low DNA damage and a more variable response at higher doses (Fig. 3D, top 7 cell lines). The remaining cell lines were more heterogeneous, showing either a sustained response to DNA damage (A498 and SKMEL5), a single non-oscillatory pulse (LOX-IMVI and HCT116), or mixed low-amplitude dynamics (MALME3). Focusing on the seven lines that showed clear oscillatory behavior, we noted that most of them (6/7), like A549 cells, showed decreased oscillations and increased amplitude of p53-YFP at higher IR doses (and presumably greater extent of DNA damage), largely converting to a single broad peak by 8Gy.

To quantify the periodic behavior of p53 in each cell line, we computed autocorrelation functions for each cell, and averaged them to derive the mean periodicity of a cell line (Fig. 3E). The seven visually oscillatory lines showed a peak in the autocorrelation function at ~5 hours, consistent with previous reports for p53 oscillatory frequency⁶. The uniformity of oscillation frequency suggests that common feedback loops govern the behavior of each cell, consistent with the universal induction of the feedback regulator MDM2 we observed (Fig. 2). This analysis also recapitulated the loss of periodicity in cell lines as IR dose increases, with some cell lines gradually transitioning from a principally oscillatory response to a more sustained response (A549 and U2OS), and other cell lines showing a stark transition at 6Gy

to a more sustained behavior (UO31, UACC257, and H460). Uniquely in this dataset, MCF7 cells become more pulsatile over time, with cells treated with 1Gy showing very little oscillatory behavior and higher doses producing increased periodicity.

In addition to loss of periodicity, increased doses of IR resulted in broader pulses of p53 activity in some cell lines. We computed the Full-Width at Half-Maximum (FWHM) of the first p53 pulse for each cell line across doses (Fig. 3F). A few lines (such as MCF7 and UACC257) showed relatively little change in FWHM with dose, whereas many of the lines that showed reduced oscillations at high doses showed dose dependent increases in FWHM (H460, A549, and U2OS). Additionally, the cell-to-cell variability of FWHM varied markedly between cell lines, ranging from exceptionally constrained (MCF7) to fairly broad (A549– and U2OS).

We further classified the twelve cell lines according to their dose dependent periodicity by comparing the periodicity of each line at 1 and 8Gy IR (Fig. 3G). We found that low periodicity lines are not greatly affected by the IR dose (blue group), whereas high periodicity lines tend to show reduced oscillations at high doses (green group). The exception was MCF7 which, as noted earlier, becomes *more* pulsatile as IR dose increases. These results underscore the complexity of p53 dynamics and their dependence on cell line identity, extent of DNA damage, and cell-to-cell heterogeneity.

Small molecule kinase inhibitor screen identifies modulators of p53 dynamics

Our results suggest that the p53 signaling in human cancer lies on a continuum ranging from pulsatile to linear accumulation. Because of the large number of genetic lesions in each line studied here, we lacked the statistical power to infer which mutations or modifications might contribute to the differential dynamics of p53 across cell lines. We therefore sought to identify modulators of p53 dynamics by screening for small molecules that alter p53 dynamics in one cell line. We used the U2OS p53 reporter line, which was intermediate in p53 response dynamics with both pulsatile (at low IR) and more sustained dynamics (Fig. 3D), and showed low cell movement making it suitable for automated analysis. The reporter line was plated in 96 well plates, pre-treated with a selection of small molecules for 2 hours, then treated with NCS to induce DNA damage, and imaged for 24 hours (Fig. 4A). Automated analysis identified ~100–200 cells in each well capable of being tracked for the duration of the movie for an average of ~15,000 single cell traces per experiment. To explore cell signaling space we used a collection of 178 small molecule kinase inhibitors from the Library of Network-Based Cellular Signatures (LINCS) consortium (table S1).

Analysis of our screening data revealed three major classes of p53 modulators: accelerators, which increased p53 abundances markedly [namely, inhibitors of CDK, focal adhesion kinase (FAK), and MDM2]; mild enhancers, which increased p53 abundances moderately [inhibitors of DNA-dependent protein kinase (DNA-PK), poly (ADP-ribose) polymerase (PARP), and glycogen synthase kinase 3 β (GSK3 β)], and dampeners, which reduced p53 and caused the cells to show a more pulsatile behavior [inhibitors of ATM and mammalian target of rapamycin (mTOR)] (Fig. 4B and table S1).

We first explored the role of CDK inhibitors on p53 dynamics. Relatively high concentrations (2 μ M) of seven of the eight CDK inhibitors we tested stabilized p53-YFP abundance in U2OS cells (Fig. 4B). The CDK inhibitors roscovitine and flavopiridol were previously reported to increase p53 concentrations due to reduced transcription of *MDM2*, likely through inhibition of CDK7 or CDK9, which are part of the general transcriptional machinery^{14,15}. Inhibition of FAK led to similar p53 behavior as observed after CDK inhibition (Fig. 4B), suggesting that FAK inhibitors may also induce p53 through loss of *MDM2* transcription. Indeed, qPCR measurements showed that abundance of the *MDM2* transcript dropped more than 3-fold after treatment with flavopiridol or PF-431396 (an FAK inhibitor) (fig. S3A).

To investigate the putative effect of CDK and FAK inhibitors on *MDM2* transcription and subsequently on p53 induction, we deployed a reporter for measuring *MDM2* transcription in live cells¹⁶ (*MDM2::T2A-GFP*) and quantified its dynamics together with that of p53-YFP in MCF7 cells. Both flavopiridol and PF-431396 led to an increase in p53-YFP, likely due to depletion of *MDM2* (Fig. 4, C and D). Once the drugs were washed out *MDM2* transcription increased rapidly, followed by depletion of p53 (Fig. 4, C and D). These drugs both acted with a delay when added or washed out compared to the direct *MDM2* inhibitor Nutlin3A, diagnostic of their slower transcriptional mechanism of action. These results show that CDK inhibitors and FAK inhibitors likely activate p53 through inhibition of *MDM2* transcription.

As opposed to CDK and FAK inhibitors, mTOR and ATM inhibitors dampened p53 activity in our screen (Fig. 4B). ATM is well known to play a major role in the induction of p53 in response to DNA damage^{17,18}, and we have thoroughly investigated the role of ATM on p53 dynamics across cell lines (see below). With regards to mTOR, we first verified that, as in U2OS cells, mTOR inhibitors lead to suppression of p53 in a second cell line (A549; Fig. 4E). Next, because mTOR and ATM are from the same family of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) kinases we asked whether the mTOR inhibitors we used might also inhibit ATM activity in this context. Focusing on the highly specific clinical mTOR inhibitor AZD8055, we observed little inhibition of Checkpoint kinase 2 (CHK2) phosphorylation at doses that had substantially altered p53 abundance (fig. S3B and table S1), arguing that perturbation of p53 dynamics by the mTOR inhibitor is not mediated through the ATM/CHK2 pathways. One other aspect of loss of mTOR signaling is a general translational attenuation. Indeed, cells treated with AZD8055 had decreased basal abundance of p53 1 hour after treatment, consistent with a translational block (fig. S3C). Such general translational attenuation may explain the damping of p53 activation we observed in mTOR-inhibited U2OS and A549 cells after DNA damage (Fig. 4, B and E). Although a specific link between mTOR signaling and p53 regulation would be intriguing, and such an interaction has been suggested¹⁹, we are currently unable to decouple the potential specific activity of TOR inhibition on p53 from its general translational effects.

The fraction of unrepaired DNA breaks varies between cell lines

We next focused on the effect of inhibiting DNA-PK, PARP, or GSK3 β on p53 dynamics. We initially confirmed that, as in U2OS cells, these inhibitors increase p53 in a second cell

line (A549; Fig. 5A). The three pathways targeted by these drugs have been implicated in DNA repair; DNA-PK and PARP inhibitors were shown to directly affect DNA repair, largely through non-homologous end joining and single strand break repair, respectively^{20–22}, whereas the effect of GSK3 β was suggested to be more tangential, possibly through p53^{23,24}. DNA-PK inhibitors also alter p53 dynamics²⁵. We confirmed that inhibition of PARP with the clinical small molecule olaparib substantially increased DNA damage measured by γ H2AX intensity 6 hours after 10Gy IR exposure compared to cells treated with IR alone (Fig. 5B; pValue <0.05 T-test). These results link the repair of DNA damage to p53 dynamics, suggesting that some of the variation we observed in p53 signaling dynamics across cell lines (Fig. 3D) might result from differences in DNA damage repair capacity. We therefore systematically measured repair capacities in our collection of 12 cell lines (Fig. 5, C–F, and fig. S4A). We found that the extent of DNA damage remaining at 24 hours after NCS treatment varied greatly between cell lines (Fig. 5, C–F). Some cell lines, such as MCF7 and U2OS, showed almost a complete repair with only a small fraction of DNA damage (γ H2AX signal) persisting (<10%). Other lines such as HCT116 or A549 showed incomplete repair, with DNA damage stabilizing at roughly 30% its peak value and persisting for at least 24 hours (Fig. 5, D and F, and fig S4A).

We next tested if the unrepaired fraction of DNA damage was a dose dependent phenomenon by measuring the fraction of DNA damage remaining after 0.5 and 8 hours across an 8 point titration of NCS treatment. We observed that the majority of cell lines show a linear correlation between their maximal DNA damage and final DNA damage (Fig. 5E and fig. S4B). This result suggests that a fixed fraction of DNA damage is slow or difficult to repair for the cell and that the fraction of damage remaining is a dose independent metric of cellular repair capacity for each cell line.

ATM activity varies greatly across cell lines

In addition to DNA repair inhibitors, ATM signaling also emerged from our screen as a major element shaping p53 signaling dynamics. The kinase ATM has been shown to phosphorylate p53 and MDM2, leading to stabilization of the p53 protein^{26,27}. We therefore asked if ATM activity plays a role in shaping p53 dynamics. We assessed ATM activity in each cell line after DNA damage by titrating the specific and stoichiometric ATM inhibitor (KU55933), measuring the resulting pCHK2 abundances and normalizing them to no ATMi conditions in each cell line (Fig. 6A). This assumes similar drug penetrance in each cell line, and uses the inhibitory concentration as a proxy for the quantity of ATM activity. Using this metric we found that ATM activity after DNA damage varies widely across cell lines with our most pulsatile cell line, MCF7, showing low ATM activity, and less pulsatile lines, such as U2OS or A549, showing stronger ATM activity (Fig. 6, A and B).

Therefore we tested whether reducing ATM activity by treatment with ATM inhibitors could broadly convert sustained lines into displaying pulsatile behavior. Indeed, treatment with moderate concentrations of ATM inhibitor followed by DNA damage altered endogenous p53 dynamics in several cell lines, rendering them more pulsatile (for example A549, A498 and UACC257; Fig. 6C). We further explored the dynamics of p53 in single A549 cells and found that single cells became pulsatile after DNA damage in the presence of moderate

ATM inhibition (Fig. 6D). In contrast, when we increased ATM signaling in the normally highly pulsatile MCF7 cells by treating them with an inhibitor of a critical ATM phosphatase, PPM1D, we observed extended and less pulsatile p53 signaling (Fig. 6E), consistent with previous results using genetic perturbation of PPM1D¹⁵. These results suggest that ATM activity is a crucial regulator of p53 dynamics and explains the non-pulsatile p53 behavior observed in some lines at higher DNA damage doses.

The variation in DNA repair efficiencies and ATM activities can explain the dynamical space of p53 across cell lines

To codify our insights into the regulation of p53 dynamics by DNA repair and ATM signaling we constructed a model of p53 signaling and ran simulations with a range of ATM activities and DNA repair efficiencies (Fig. 7A). Consistent with our experimental observations, our model showed periodic signaling in a wide range of conditions, with low ATM activity or high DNA repair efficiency resulting in a single or a few pulses. Moderate ATM activity sustained p53 oscillations, and high ATM activity led to a much broader p53 peak. DNA damage dose moved cells along the ATM signaling axis, but note that repair efficacy is an intrinsic property of a cell line that is unaffected by dose (fig. S4). The combination of DNA repair efficiency and ATM activity therefore creates a ‘signaling space’ in which different cell lines may occupy different regions (Fig. 7B). We suggest that a cell line’s starting point in this signaling space will result in qualitatively different p53 signaling patterns in response to different doses of DNA damage. For example, A549 cells show moderate repair but high intrinsic ATM activity, which at high DNA damage doses pushes this cell line out of the oscillatory p53 signaling regime and into more sustained behavior. MCF7 cells on the other hand have low ATM activity and do not leave the oscillatory regime even at high DNA damage doses (Fig. 7B). Examining p53 amplitude in this signaling space shows that within the oscillatory regime a range of p53 amplitudes are possible, suggesting that gene expression regulated by dynamics and amplitude could co-exist (Fig. 7B).

Discussion

In response to DNA damage p53 abundance rise rapidly, triggering a cascade of transcription enforcing cell cycle arrest and, if the damage is severe enough, committing the cell to a terminal fate. Ionizing radiation was shown to trigger oscillations of p53 in MCF7 cells, in the non-transformed RPE1 cells, and in live mice^{6,9,10,15}. These oscillations were shown to depend on the feedback loop with the E3 ligase, Mdm2. As p53, MDM2 and the upstream kinase regulators Chk2 and ATM are among the most frequently mutated genes in cancer, we asked how the dynamics of the p53 pathway might have diverged in tumor lines. Focusing on twelve lines expressing wild-type p53, which we showed are capable of regulating MDM2 transcription and therefore imposing feedback regulation, we investigated the variation on p53 dynamics across lines in response to DNA double strand breaks.

While some cell lines showed stable oscillations of p53, like MCF7 cells, others showed diverse behaviors from broad peaks to slow ramps. Further, we observed that while some cell lines have invariant behavior regardless of DNA damage dose, others are sensitive to the magnitude of damage and show both different duration of p53 activation and different

dynamical patterns of p53 under different doses. Some dynamical behaviors were however highly conserved across all cell lines, most notably the fundamental period of p53 oscillations, which was a stable ~5hrs for all cell lines with detectable oscillations. This result constrains our understanding of how the p53 network varies across systems, arguing that the feedback circuit driving p53 oscillations is not greatly affected by tissue context, but that upstream regulation of p53 signaling through ATM or other DNA damage signaling is.

Chemical and genetic screens have been previously used to look at the dynamics of circadian rhythms, leading to the identification of regulators responsible for maintaining the circadian period and amplitude^{27,28}. Here we applied a similar live single cell screening approach to identify kinase inhibitors that modify p53 dynamics. We identified several broad classes of inhibitors that modified p53 signaling, including ATM inhibitors, which converted p53 signaling from a broad peak to oscillations. This approach allowed us to examine the interaction between small molecule inhibitors and DNA damage at high resolution. As the dynamics of p53 signaling encode important information about both the nature of DNA damage and also cell fate, such high resolution is critical. We suspect that as technology for assaying molecular events inside cells, and for high-throughput microscopy improves such screens will become increasingly common.

Our small molecule screen also revealed that inhibitors of mTOR, CDKs, and PARP, all pathways targeted by cancer drugs currently in the clinic, also altered p53 activity. They appear to act through a mixture of DNA damage repair and global effects on transcription and translation. Notably, CDK inhibitors increased p53 abundance, likely by blocking MDM2 transcription¹⁸, and although the p53 generated by CDK inhibition is likely harmless (as it cannot induce transcription in the presence of the drug), when the CDK inhibitor is washed away, p53 rapidly activates downstream targets to levels comparable to specific MDM2 inhibitors, suggesting that the activity of these compounds on p53 may depend greatly on pharmacokinetics of the specific small molecules.

Clinically applying combinations of DNA damage and small molecules to modify p53 signaling dynamics and treatment efficacy will require tumor specific predictive modeling integrating the genotype of the tumor and the mechanism of each treatment modality. The model we developed here showed that depending on where a cell line starts in p53 signaling space, perturbations of ATM or DNA repair can result in different signaling outcomes. These results suggest that careful measurements of features such as ATM activity at baseline conditions may enable rational design of combination therapies to achieve specific cellular signaling dynamics in any given tumor.

Comparing p53 dynamics across tumor lines revealed an unexpected diversity of responses. Our work suggests that other dynamic regulatory signals which were shown to have stereotyped behaviors (for example NF- κ B or ERK), may also vary substantially across cell lines, tumors and tissues in the body. Combining single cell reporters, batch immunofluorescence, and medium throughput screening as we used here to measure p53 dynamics across a diverse set of cell lines, will be essential for determining the conservation of dynamics in other signaling pathways and the machineries that permit this diversity.

Materials and Methods

Cell culture

Parental cell lines were obtained from ATTC, thawed and propagated in RPMI (GIBCO) with 5% FBS. All experiments were performed in this media. For microscopy RPMI lacking phenol red and riboflavin was used. For viral production 293T cells were grown in DMEM (GIBCO) + 10% FBS. All media was supplemented with 1% antibiotic and antimycotic (Corning). Cells from each line were infected with p53-YFP lenti virus in 35mm dishes, selected with G418 (sigma) of varying concentrations, and split into 96 well plates to select for clones expressing p53-YFP.

Immunofluorescence

Cells were plated in 96 well flat bottom plates (Corning), grown for 24–48hrs, treated as indicated, and fixed with 2% paraformaldehyde (PFA, Alfa Aesar) for 10 minutes. Plates were washed twice with Phosphate Buffered Saline (PBS) and permeabilized with 0.1% triton X-100, before sequential staining with primary and secondary antibodies. Cells were washed three times with PBS and imaged within 24hrs.

Virus production and infection

Virus was produced using 293T cells transfected with p53-YFP constructs and viral packaging vectors and viral supernatant were collected after three days. For viral infection, Cells were plated at low density and cells were infected with virus in media containing HEPES and protamine sulfate. Cells were allowed to recover in nonselective media for one day. Cells productively infected were selected with the appropriate antibiotic.

RNA extraction and qRT-PCR

Fifty-Thousand cells were plated in 6 well plates. Cells were cultured for 24hrs and then treated with the indicated compounds. RNA was extracted by treatment with Trizol and subsequent purification on a Zymo RNA column. The bulk RNA was reverse transcribed using the high capacity reverse transcription kit (applied biosystems) to produce cDNA. Transcript abundance was quantified by specific primers for MDM2 using a Sybr green (lifetechnologies) based qPCR and normalized to Actin.

Antibodies and Reagents

Primary antibodies for p53 (FL393 -- Santa Cruz biotech (SCB)), MDM2 (SMP14 SCB), pCHK2 (Cell signaling), γ H2AX (JBW301, Millipore), p21 (Calbiochem) and Actin (Sigma) were obtained and used at 1:400–800. Secondary goat anti-mouse or anti-rabbit antibodies conjugated to AF555 or AF647 were purchased from Invitrogen. Small molecules were purchased from Enzo life sciences (Flavopiridol [CDK1i]), Sigma (CH99021, NU7026 [DNA-PKi], Neocarzinostatin [NCS]), Biovision (AZD8055[TORi]), and Calbiochem (KU55933 [ATMi]), DAPI (Life Technologies), EMDmillipore (GSK2830371[PPM1Di]). X-ray induced DNA damage was generated with a RS-2000 source (RadSource).

Microscopy

For live cell imaging cells were plated in glass bottom 35mm dishes (Matek) 24–48hrs before imaging, 1–2hrs before imaging cells were switched to transparent media (RPMI lacking riboflavin and phenol red, Invitrogen). Live cell imaging was performed with Nikon TI microscope equipped with a heating chamber and CO₂ source, an epi-fluorescent source (either mercury arc lamp (Prior) or LED system (Lumencor)), automated stage (Prior), YFP filter set (Chroma) and CCD or CMOS camera (Hamamatsu).

Fixed microscopy was performed using a Nikon TI microscope equipped with an epi-fluorescent source (either mercury arc lamp (Prior) or LED system (Lumencor)), automated stage (Prior), Cy3, Cy5 and DAPI filter sets (Chroma) and CCD or CMOS camera (Hamamatsu).

Small Molecule Screening

U2OS cells expressing p53-YFP and RFP-NLS were plated in plastic 96 well flat bottom plates (corning). Cells were grown for 24hrs and switched to transparent media. Drugs were applied using an automated pinning system at the ICCB Longwood screening facility. Plates for live imaging were then placed on a microscope and imaging positions determined. Imaging began immediately to determine a pre-NCS baseline 90–120 minutes after drug addition. NCS was added to each well and the plates were imaged for 24hrs. Replicate plates were treated with NCS, left in the incubator for 10hrs, fixed with PFA and subsequently stained for p53, imaged, and the antibody signal was quantified.

Microscopy data analysis

Microscopy data (live and fixed) was processed with custom MATLAB code. Single cells were tracked manually using the phase images with a MATLAB interface. Single cell tracks were projected onto the fluorescent images, which were then background corrected (by Median filtering and subsequent tophat background subtraction) and nuclear signal (estimated as the average of the top 10 pixels in the nuclear area) was then computed for the applicable channels. For fixed images, the images were segmented using the DAPI channel with a watershed algorithm and mean nuclear intensity computed for each cell. Images in displayed in the body of the paper were smoothed with a median or Gaussian filter and background subtracted. Contrast was adjusted for optimal visualization and is consistent between pre- and post-treatment for all images. Automated tracking of U2OS cells was preformed using a nuclear marker. Nuclei were identified in a given frame as objects with a given mCherry intensity, shape and size. p53-YFP intensity was computed as the mean of the brightest 10 pixels within an identified region. Identified cells were connected across frames using a greedy nearest neighbor algorithm resulting in single cell traces.

Computational Modeling

We constructed a four species, 18 parameter model describing p53 oscillations driven by MDM2 transcription based feedback using a delayed-differential equation (DDE) framework. The two principal species, MDM2 and p53, were present in phosphorylated and non-phosphorylated forms. The phosphorylated MDM2 and p53 species had increased or

decreased degradation rates respectively. We performed all simulations in MATLAB (see supplemental text for details).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. p53 abundance and the initial response to DNA damage are comparable across cancer cell lines

(A) Twelve cell lines were stained for p53 before or 2 hours after treatment with the DNA damaging agent NCS (100 ng/ml). Scale bar, 50 μ m. “M” marks the 5 melanoma lines. (B) Histograms of single cell staining intensity of p53 before (blue) or after (red) treatment with NCS. Black lines indicate the medians of the distributions. Cells are ordered by their median p53 abundance after NCS. (C and D) A549 cells were stained for p53 2 (C) or 8 (D) hours after a log2 titrations series of NCS doses. Red lines indicate the median of the distribution. The abundance of p53 did not vary significantly across DNA damage doses at 2hrs (pvalue >0.05), but did after 8hrs (pvalue <0.05, unpaired ttest). (E and F) Each cell line was stained for p53 2 (E) or 8 (F) hours after varying doses of NCS. Heatmaps represent p53 abundance. Each cell line was internally normalized (Norm.) between 0 and 1. Data in (B–F) are from N>500 cells for each cell line, representative of 2 independent experiments. AU, arbitrary units.

Figure 2. p53 shows different temporal patterns across cell lines

(A and B) Abundance of p53 and MDM2 was quantified by immunofluorescence and are plotted as boxplots showing the change () in protein abundance between the 0 and 2-hour time points or between the 2 and 8-hour time points after NCS treatment (100 ng/ml). Dots represent each of the 12 cell lines, highlighting here MCF7 (green), A549 (red), and HCT116 (blue). (C) Abundance of p53 quantified by immunofluorescence at the indicated time points after NCS treatment. Histograms from the three cell lines are shown. Data in (A–C) are from $N > 500$ cells for each cell line; representative of 2 independent experiments.

Figure 3. Live cell tracking of p53 over time reveals cell line specific dynamics and dose dependency

(A) Images of MCF7 and A549 cell lines expressing p53-YFP over 18 hours after 1 or 6Gy IR exposure. Scale bar, 50 μ m. (B) Heat maps of p53-YFP abundance in MCF7 and A549 cells after exposure to various doses of IR. Each row represents a single cell. (C to F) Twelve cell lines were constructed to express p53-YFP and imaged after exposure to IR: 1Gy, blue line; 2Gy, red line; 4Gy, yellow line; 6Gy, purple line; 8Gy, green line (or box plot in F). Data are from N>50 cells for each condition (3825 cells in the full dataset, pooled from 2–3 experiments for each line). ‘M’ indicates melanoma cell lines. Measured for each dose in each cell line was (C) the fraction of cells which have divided at a given time; (D) p53-YFP abundance (bold colored lines, averages; grey lines, single cell traces); (E) average autocorrelations of p53 trajectories; and (F) the Full-Width-Half-Maximums of the first p53

pulse (box plots show the distribution over single cells; * indicates cell lines where the FWHM is dose-dependent; $P < 0.05$ by a t -test). **(G)** A measure of periodicity (maximum of autocorrelation function minus the minimum of autocorrelation function in the first 5 hours; see inset) was calculated for each cell line and condition.

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Figure 4. A kinase inhibitor screen in live cells identified compounds that alter p53 dynamics after DNA damage

(A) Screen workflow in which U2OS cells expressing p53-YFP were incubated for two hours with kinase inhibitors then exposed to NCS (100ng/ml), followed by live cell imaging of p53-YFP abundance over 24 hours. (B) Effect of pretreatment with various signaling molecule inhibitors on NCS-induced p53-YFP dynamics in U2OS cells. In the control graph (DMSO; far left), the bold blue line is the average trace and the gray lines are single cell traces. Each graph then shows the control trace as a reference (bold blue trace) alongside colored traces representing the average trajectory of p53-YFP abundance in cells exposed to specific compounds within each class of drug (each of 8 CDK inhibitors, 2 FAK inhibitors, 4 mTOR inhibitors, 3 ATM inhibitors, and 4 distinct combinations of 2 PARP and 2 DNA-PK inhibitors.). Bottom, heat maps of p53-YFP abundance in single U2OS cells after NCS (100ng/ml) treatment and the corresponding drug treatment (color-coded groups correspond to the traces above). (N>20 cells for each condition). (C and D) Abundance of p53-YFP (C) or the transcriptional reporter MDM2::T2A-GFP (D) in transfected MCF7 cells exposed to a

three-hour pulse (shaded) of the MDM2 inhibitor nutlin-3A, the CDK inhibitor flavopiridol, or the FAK inhibitor PF-431396. **(E)** Abundance of p53-YFP in A549 cells treated with either 100ng/ml NCS (top; single cell traces in gray; average in bold) or the mTOR inhibitor AZD8055 then NCS (bottom; blue NCS-only trace inserted for reference). Data in (C–E) are from N>25 cells; representative of 2 exp.

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Figure 5. Repair proficiencies vary between cell lines

(A) A549 cells expressing p53-YFP were pre-treated with DMSO (NT) or Olaparib (PARPi; 10uM), NU7026 (DNA-PKi; 10uM), or CH99021 (GSK3 β i; 10uM) for 1hr and then treated with NCS (100 ng/ml). Red lines represent the treated condition (thin lines, single cells; thick line, average), and the blue line represents the control (NT) cells (N>40 cells, representative of 2 independent experiments.). (B) Quantification of γ H2AX intensity induced by NCS (100ng/ml; DNA damage) was measured in A549 cells after 1hr pretreatment with DMSO (NT) or the indicated inhibitor (10uM) 6 hours after treatment with 10Gy IR. Scale bar, 25 μ m. Histograms are shown, with a red line indicating the median (N>500 cells, representative 3 independent experiments.). (C) Images of cells from the indicated lines stained for γ H2AX before or 0.5 or 24 hours after NCS treatment (100ng/ml). (D) Histograms of MCF7, A549 and HCT116 cells stained for γ H2AX at the indicated time-points after NCS treatment (N > 200 cells from 2 independent experiments.). (E) γ H2AX in HCT116 cells was quantified 30 min and 8 hours after NCS treatment. (F) DNA damage assessed as γ H2AX abundance at 30 min and 8 hours in the indicated cell lines were compared across a range of NCS concentrations (noted in the color scale). Data are means $\pm$ S.D. (N=7 doses).

Figure 6. ATM signaling varies across cell lines and manipulation of ATM modifies p53 periodicity

(A) ATM activity was quantified by immunofluorescence measurements of phosphorylated (p) CHK2 30 min after 100100ng/ml NCS treatment in the presence of one of 8 doses of ATM inhibitor KU55933. Data is shown as a lineplot, smoothed with a window of 3. (B) The IC₅₀ of each cell line in response to ATM inhibition calculated from (A). (C) Mean p53-YFP abundance measured by immunofluorescence in each cell line treated (red) with ATM inhibitor (2 μ M), or DMSO (black), and at 2, 5, and 7 hours after 100ng/ml NCS treatment (N>200 cells, representative of 3 experiments). (D and E) Left, p53-YFP abundance in A549 cells exposed to 6Gy IR (D) or MCF7 cells exposed to 8Gy IR (E) in the presence (bottom) of ATM inhibitor (KU55933; 2 μ M; D), PPM1D inhibitor (2 μ M; E), or DMSO (top). Right, corresponding average autocorrelation curves (N>50 cells, representative of 2 independent experiments).

Figure 7. ATM signaling and DNA repair capacity provide a range of p53 dynamical behaviors (A) A computational model of p53 signaling was simulated with varying ATM activities and DNA repair efficiencies, resulting in a range of p53 dynamics capturing the experimental measurements in the different cell lines. (B) The maximum p53 amplitude and periodicity are shown as heat maps for various values of ATM activities and repair efficiencies. The putative ‘signaling spaces’ in which MCF7 or A549 cells may reside are indicated, as are the effects of chemical inhibitors used in this study (right).