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14. ABSTRACT

The p53 transactivation domain 1 (TAD1) plays a critical role in inducing p53 mediated cell-cycle arrest and apoptosis in response to acute DNA damage caused by irradiation. During radiation therapy of cancers, this p53-induced apoptosis triggers various deleterious pathological side effects in normal tissues. Interestingly, recent studies from our laboratory have demonstrated that p53 TAD1 is completely dispensable for tumor suppression in diverse mouse cancer models. We hypothesize that specific inhibition of p53 TAD1 should ameliorate the p53-associated pathologies occurring in response to acute DNA damage, while keeping p53-mediated tumor suppression intact, thus allowing improvement in the therapeutic index of radiation therapy in cancer. Importantly, because the majority of cancers, such as advanced prostate cancers, have inactivated the p53 pathway, such inhibitors should not compromise the efficacy of treating tumors. We propose to perform high-throughput chemical library screens to identify specific inhibitor of p53 TAD1 that may be administered as adjuvants of chemotherapy and radiotherapy in the context of prostate cancer.

15. SUBJECT TERMS
p53 inhibitor, p53 transactivation mutants

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Table of Contents

Page

1. Introduction……4

2. Keywords……4

3. Overall Project Summary……4

4. Key Research Accomplishments……9

5. Conclusion……9

6. Publications, Abstracts, and Presentations……9

7. Inventions, Patents and Licenses……9

8. Reportable Outcomes……9

9. Other Achievements……9

10. References……10

11. Appendices……11

Introduction

The p53 protein plays a pivotal role in suppressing tumorigenesis, as evidenced by its inactivation in over half of human cancers. Through its ability to function as a transcriptional regulator, p53 has the capacity to respond to a variety of genotoxic stress signals and induce cell-cycle arrest, apoptosis and cellular senescence to curb neoplastic growth (*1, 2*). Aside from its beneficial tumor suppressive capability, p53 is also a critical mediator of DNA damage signals and this property provokes it to induce deleterious, pathological side effects associated with genotoxic stress-inducing radio- and chemotherapies, particularly in the radiosensitive tissues. The active role of p53 in provoking the detrimental side effects of cancer therapy has opened up the possibility of developing specific p53 inhibitors that can be used as radiotherapy and chemotherapy adjuvants to reduce these side effects. It would be ideal to identify p53 inhibitors that could suppress p53-associated pathologies without perturbing p53 tumor suppressor function. Previously, we established that p53 transactivation domain 1 (TAD1) is critical for inducing cell cycle arrest and apoptosis in response to acute DNA damage but is dispensable for p53-mediated tumor suppression in various mouse cancer models (*3, 4*). Based on these observations, we hypothesize that inhibition of p53 TAD1 will selectively inhibit p53-dependent, radiation-induced cell death and subsequent normal tissue damage during radiation and chemotherapy of p53-negative cancers. Towards this end, we propose to perform a high-throughput chemical library screen to identify p53 TAD1 inhibitors by generating and using a mouse embryonic fibroblast reporter system that expresses dual color luciferase or dual fluorescent reporters under the control of p53 TAD1-dependent and independent target gene regulatory elements.

Keywords

p53 inhibitor, p53 transactivation mutants, p53 transcriptional activation domain inhibitor

Overall Project Summary

Specific Aim 1: To generate mouse embryonic fibroblasts (MEFs) stably expressing p53-inducible fluorescence reporters for screening

Our major goal is to identify p53 transactivation domain 1-specific inhibitors by generating and subjecting reporter-expressing MEFs to a chemical library screen. As described previously, we proposed to generate a dual-reporter system (click-beetle Green and Red luciferases), with one under the control of p53 responsive regulatory sequences of a p53 TAD1-dependent gene, *Cdkn1a* (*p21*), and the other under the control of a TAD1-independent gene, *Crip2*. This approach will facilitate the identification of p53 TAD1 inhibitors through a single-step luciferase assay-based high-throughput screen of small molecule libraries. As an alternative strategy, we also proposed to construct a dual-fluorescent

reporter cell line with TdTomato and EGFP under the control of the *Cdkn1a* (*p21*) and *Crip2* promoters, respectively, which could additionally be utilized to perform image-based screens. In addition, as a backup strategy, we have sought to generate two separate cell lines, one expressing the *Cdkn1a* (*p21*) promoter fused to firefly luciferase and the other expressing the *Crip2* promoter fused to firefly luciferase. The advantage of this approach is that 1) both genes will express the same reporter, ruling out any reporter-specific differences, and 2) compounds that inhibit firefly luciferase are known and can be readily eliminated from the hits. In this case, we would screen compounds first in the p21-luciferase line, and then take positive hits and repeat the screen with the Crip2-luciferase cell line. Here we present a summary of the construction and validation of these reporter cell lines as well as the results from a pilot screen for general p53 inhibitors.

Task 1a. Construct cell lines stably expressing p53 reporters, to use in chemical library screening

Generation of reporter lines in Arf-/- immortalized MEFs.

As described in detail in the previous annual report, we utilized CRISPR/Cas9 targeting strategies, which enable homology-directed repair at a locus of interest, to generate reporter expressing cell lines. We generated reporter-expressing Arf-/- immortalized MEFs expressing various reporters under the governance of p53 responsive regulatory sequences of a p53 TAD1-dependent gene, *Cdkn1a* (*p21*) and TAD1-independent gene, *Crip2*. We had proposed to generate cell lines with color luciferase, fluorescent, and firefly luciferase reporters to ensure the highest chance of ultimate success with the screen. In our previous report, we presented promising initial targeting validation data for one of these cell lines (p21-Green luciferase-Puro). In the past year, we further analyzed the entire targeted loci in all the reporter cell lines (including p21-Green luciferase-Puro, p21-GFP-Puro, p21-Red Luciferase-Puro, p21-tdTomato-Puro and Crip2-Red Luciferase-Puro) by PCR genotyping & Southern blotting of genomic DNA sequences from the targeted cell populations. Unfortunately, we uncovered a mistargeting event at the 5' DNA ends of the targeted loci in all the cell lines such that none of the lines were suitable for any further processing, prompting us to revise our targeting strategy (discussed below). Specifically, we discovered that extra DNA sequences originating from the targeting vector were inadvertently introduced into the upstream regulatory region of the *p21* or *Crip2* gene during the process of homology directed repair. Due to the strong possibility that these extra plasmid sequences might interfere with the p53 dependency of expression of the reporters, we decided to not further pursue these lines. We believe that this spurious targeting event was likely due to the CRISPR/Cas9 mediated digestion and consequent linearization of the targeting vectors, allowing integration into the *p21* or *Crip2* loci. Given the very recent development of CRISPR/Cas9 targeting, such technical issues have been found to arise, and we have taken immediate steps to revise our strategy.

To revise our targeting strategy, we devised a two-pronged approach. First, we generated variants of targeting vectors in which we introduced point or deletion mutations in the sgRNA binding site in the 3' homology arm (the PAM motif). Second, we identified new sgRNAs that bind in a region spanning the junctions of the two homology arms **(Figure 1A and 1B)**. In both of these scenarios, the CRISPR/Cas9 system should selectively target the intended genomic DNA sequences but not the targeting vectors. Using the new targeting strategy, we were able to generate a p21-tdTomato-Puro line that was validated both by 3' and 5' Southern blotting (**Figure 1C and 1D**) and PCR genotyping (data not shown). The intended targeting of the *p21* locus worked correctly only in the case where a targeting vector harboring a point mutation in the PAM motif of the sgRNA binding site in the 3' homology arm was introduced into MEFs (**Figure 1D, lane 3**). With this revised strategy, we can now readily generate the dual-reporter cell lines.

Generation of *p21-firefly luciferase* MEFs.

While optimizing the generation of reporter cell lines, in the past year we simultaneously generated MEFs from already existing *p21-firefly luciferase* knock-in mice (*5*) and subjected them to a small proof-of-concept screen. To generate *p21* promoter driven firefly luciferase expressing MEFs, we mated *Cdkn1atm1Hpw* (JAX #023429) homozygous male mice with wild-type female mice. From this mating, we recovered 8 embryos at day E13.5 and further individually processed them to generate 8 different MEF lines of heterozygous *p21-firefly luciferase/+* MEFs (p21-Fluc MEFs). We demonstrated that these MEFs displayed robust readily-detectable luciferase reporter activity that was sensitive to acute-DNA damage (**Figure 2A**). In addition, the reporter activity was found to be highly p53-dependent in p53 knock-down assays (**Figure 2B, 2C**).

As described later in this report, we have successfully utilized these MEFs to perform a proof-of-concept pilot small molecule screen of general p53 inhibitors. Through this pilot screen, we have identified several potential general p53 inhibitors that we are currently validating in *in vitro* assays.

In the past year, we also pursued generating a separate reporter cell line that expresses fluorescent or firefly luciferase reporters under the control of a TAD1-independent gene, *Crip2,* which would allow us to counter-screen for p53 TAD1-specific inhibitors out of the general p53 inhibitors that will be identified in the primary screen using p21-Fluc MEFs. We have generated a vector that can be introduced into Arf-/- MEFs.

Specific Aim 2: To conduct chemical library screen for compounds inhibiting *p21* but not *Bax* fluorescence reporter activity in wild-type MEFs upon DNA damage treatment

As described previously, we made a few technical changes to this aim to enhance the probability of success with our screens. To summarize, first, we are using a *Crip2* gene reporter instead of a *Bax* reporter as the p53 TAD1-independent reporter. Second, we are using immortal *Arf* null MEFs instead of wild-type MEFs to construct the reporter cell lines. Finally, in addition to trying to generate cells with dual color luciferase or dual fluorescent reporters, we generated cells expressing a single firefly luciferase reporter for our high-throughput screening due to the aforementioned technical problems with CRISPR targeting. Specifically, after extensive discussions with the director of the Stanford High-Throughput Bioscience Center (HTBC), we decided to run a primary screen for general p53 inhibitors using cells expressing a single *p21*-firefly luciferase reporter and then to subsequently perform a counterscreen for TAD1-specific inhibitors in cells carrying a *Crip2*-firefly reporter.

Task 1b. Conduct small-scale pilot screen to optimize conditions of screening

To conduct the pilot screen, we characterized the p21-Firefly Luciferase reporter cell line to define the optimal number of cells for screening by analyzing the reporter activity upon titrating a population of cells in 384-well plates (data not shown). We observed that as a few as 1000 cells was sufficient to detect robust luciferase activity that was responsive to doxorubicin DNA-damage treatment. We also determined the optimal time point at which to measure the luciferase activity after addition of luciferase reagent such that the luciferase readout is stable and consistent throughout the plate without any significant edge effects (data not shown).

We tested LOPAC (Library of Pharmacologically Active Compounds, Sigma-Aldrich), Microsource Spectrum (Microsource Discovery Systems, Inc), NIH Clinical Collection, Enzo Life Sciences, and FDA v2.0 libraries containing a total of 3688 unique compounds. The LOPAC library (1269 unique compounds) has an annotated collection of small molecule modulators and approved drugs that impact most cellular processes and cover major drug target classes. The Microsource Spectrum collection (1502 unique compounds) includes compounds selected by medicinal chemists and biologists to provide a wide range of biological activities and structural diversity. The NIH Clinical Collection (NCC) comprises 446 unique compounds with a history of use in human clinical trials. The Enzo Life Sciences ICCB Known Bioactives (296 unique compounds) and FDA v2.0 (175 unique compounds) libraries include collections of diverse biologically active compounds with defined biological activity and FDA-approved drugs with known bioactivity, respectively. During qHTS, these libraries were screened in a 7-point titration format and concentration-response curves were generated for each compound on *p21* luciferase reporter MEFs treated with Doxorubicin. Based on optimization experiments (data not shown), we treated with chemical compounds for 24 hours total, adding Dox for the last 6 hours of

this period, a sufficient time to induce endogenous p53 target gene expression. Inhibitory compounds classified to top high-response groups for *p21* reporter activity were selected as candidate hits for further analysis. Compounds that cause significant cytotoxicity by CellTiter-blue cell viability assays were eliminated.

Optimized protocol for performing luciferase assay based high-throughput chemical library screening using p21-Fluc MEFs

For the pilot screen, we plated 2000 cells per well (50 µL) in solid white 384-well plates and black-clear bottom 384-well plates. The plates were placed into the automated incubator and after 16-24 hours incubation at 37^{o}C, 5% CO_2 the Staccato System removed the plates from the incubator and added 100 nL of compounds using the Pin Tool. After 18 hours incubation at 37^{o}C, 5% CO_2, 10 µL of 1.2 mg/mL of Doxorubicin (DOX) was added to both the white and black plates to induce DNA damage. 10 µL of Cell-Titer Blue Reagent (CTB, 1:2 dilution in DMEM media) was added to the black plates with the Wellmate robot. The plates were returned to the incubator for another 6 hours before adding 10 µL of Brite-Glo Luciferase Reagent (1:2 dilution in PBS) to the white plates with the offline multidrop robot and read in the Tecan Infinite M1000 PRO plate reader after ~30 minutes. The black plates were read in the Tecan Infinite M1000 PRO at 6 hours after DOX and CTB addition and after 20-24 hours. Initial hits were defined as compounds that inhibit the luciferase activity compared to no compound controls, and have been shown to be non-toxic in the CTB assay and other cell line screens performed at Stanford HTBC.

Results from qHTS screening of p53 inhibitors

We identified ~50 compounds that inhibit luciferase activity without compromising cell viability by CellTiter-blue assays, demonstrating our ability to identify inhibitors of *p21* expression (**Figure 3**). While we still need to formally validate which compounds act by inhibiting p53 activity, *p21* expression in MEFs is highly p53-dependent, suggesting we will have some p53 inhibitors. This effort clearly established confidence in our assay, supporting the idea of pursuing a High-Throughput Screen as a next step. We will conduct the full-scale chemical library screen to maximize the discovery of optimal p53 TAD1 inhibitor(s). The >130,000 compounds at Stanford include diverse compound libraries (e.g. ChemDiv, SPECS and Chembridge) as well as specific libraries (ie. kinase-targeting libraries). Unlike the libraries in the small-scale screen, the compounds in the large-scale libraries have unknown bioactivities and physico-chemical properties and have been selected based on computational methods to identify diverse structures with a range of drug-like and lead-like properties.

Specific Aim 3: To confirm that the candidate compound(s) inhibits p53-dependent acute DNA-damage responses and improves prostate cancer treatment in mouse models

No changes.

Key Research Accomplishments

Nothing to report

Conclusions

Our laboratory has provided the first identification of a mechanism distinguishing p53 action in acute DNA damage responses and in tumor suppression, which we leverage here to identify compounds that could be used to mitigate side effects of prostate cancer therapies without causing risk of additional cancer development. Identification of TAD1 inhibitors could allow protection from radiation-induced toxicity to normal tissue, thereby allowing enhanced doses of radiation to be administered and enhancing prostate cancer treatment. The experiments described above are critical initial steps for identifying such TAD1 inhibitors.

Publications, Abstracts and Presentations

Nothing to report

Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

References

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3. C. A. Brady *et al.*, Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* **145**, 571 (May 13, 2011).
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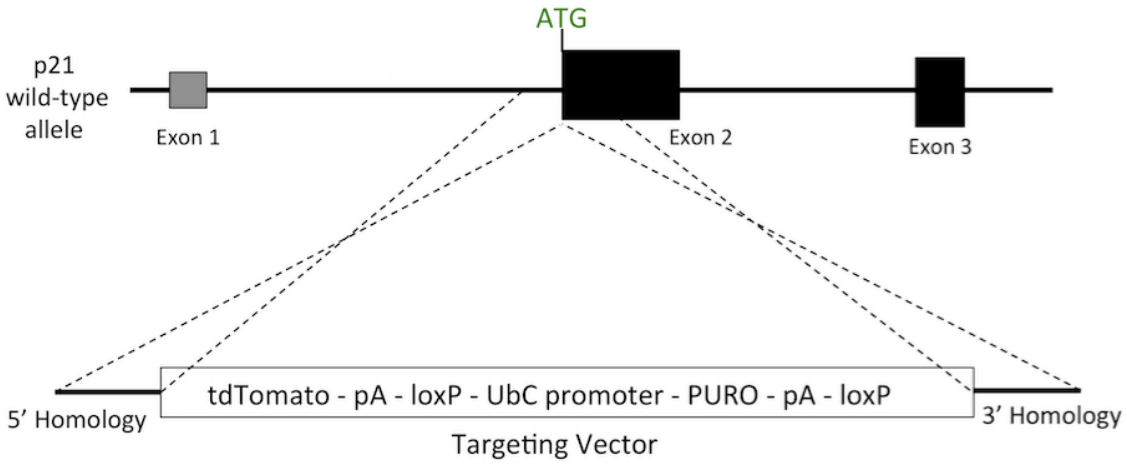
5. K. L. Tinkum *et al.*, Bioluminescence imaging captures the expression and dynamics of endogenous p21 promoter activity in living mice and intact cells. *Molecular and cellular biology* **31**, 3759 (Sep, 2011).

Appendices

Figure 1

A



B

		Target sequence	+ PAM	
p21 CRISPR guide 1:	5'-	GATGTCCGACCTGTTCCGCA	CAG	-3'
p21 CRISPR guide 2:	5'-	CTAGGCTCCGAACGCGCTCC	CAG	-3'
p21 CRISPR guide 3:	5'-	CCGTGATTGCGATGCGCTCA	TGG	-3'
p21 CRISPR guide 4:	5'-	ACAGGCACCATGTCCAATCC	TGG	-3'
p21 CRISPR guide 5:	5'-	GACATCACCAGGATTGGACA	TGG	-3'

Fig. 1A. Targeting scheme used to generate p21-tdTomato-Puro MEF line. Dotted gray lines indicate the targeted sites for homologous recombination around the p21 translation start site in Exon 2 **(B)** p21 CRISPR guide RNA sequences identified by the MIT CRISPR design tool. CRISPR guides 1,2 and 3 target both genomic sites and the 3' homology arm in the targeting vector. CRISPR guides 4 and 5 target only genomic sites as they bind to a sequence spanning the junction of the homology arms.

Figure 1

C

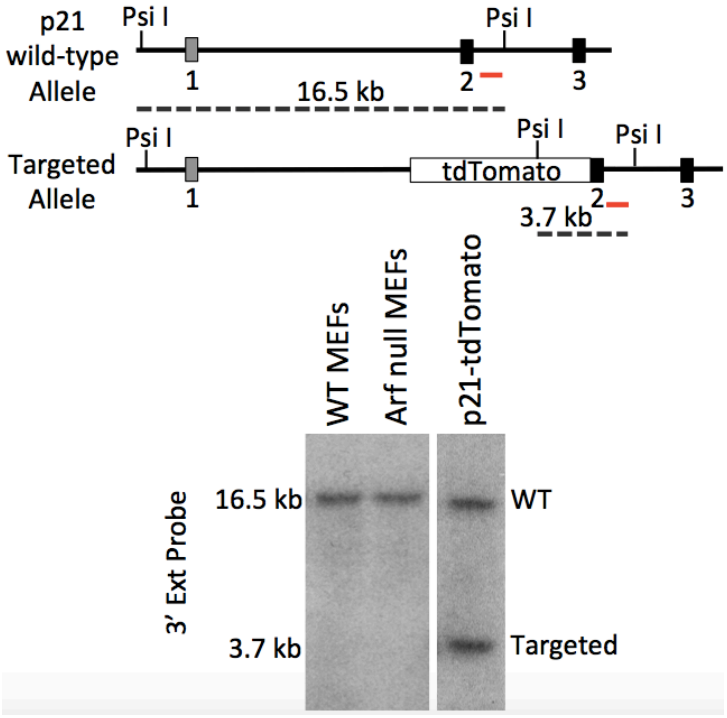


Fig. 1C. Southern blot analysis of MEF cell line expressing tdTomato under the control of endogenous *p21* promoter. Genomic DNA digested with PsiI and Southern blot probed with the 3' external probe (red).

Figure 1

D

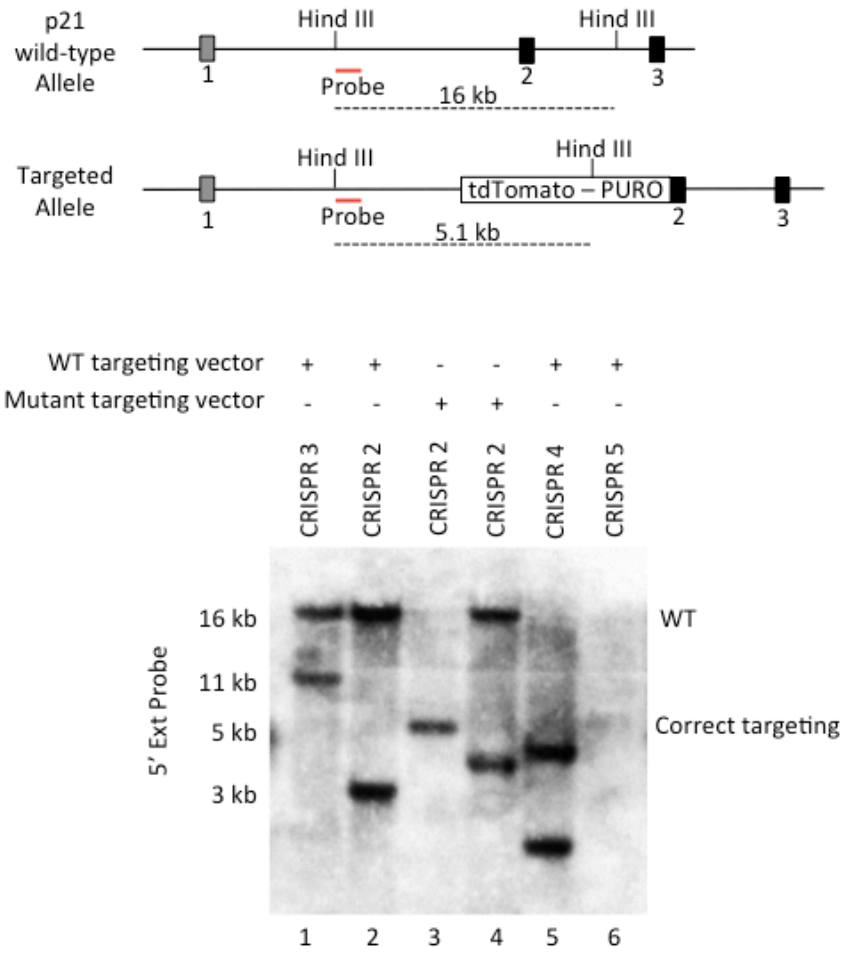


Fig. 1D. Southern blot analysis of MEF cell lines expressing tdTomato under the control of endogenous *p21* promoter. Genomic DNA digested with HindIII and Southern blot probed with the 5' external probe (red). Sample in lane 3 was from cells in which CRISPR guide 2 was introduced together with targeting vector harboring point mutation in the PAM motif while in lane 4, the entire sgRNA binding site was deleted.

Figure 2

A

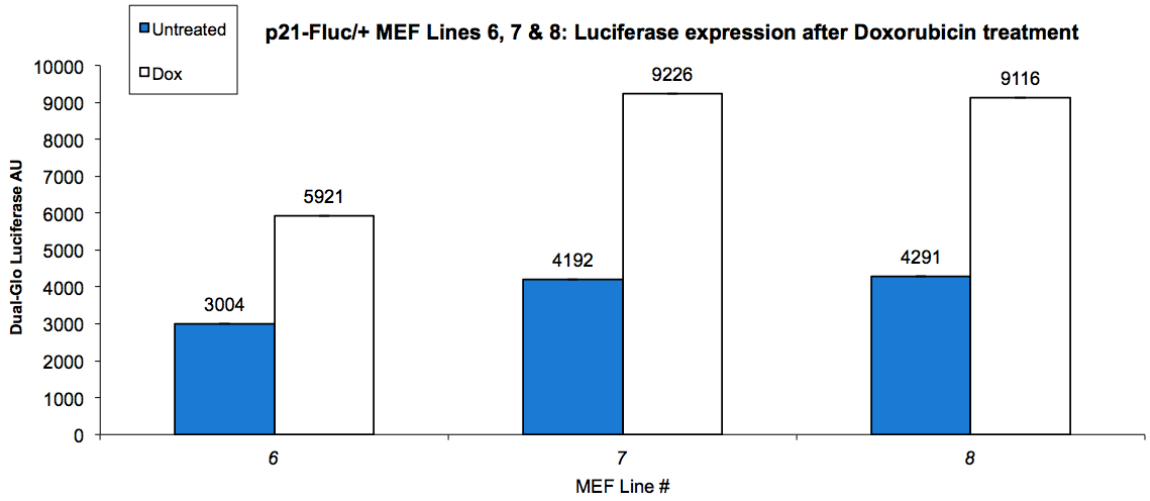
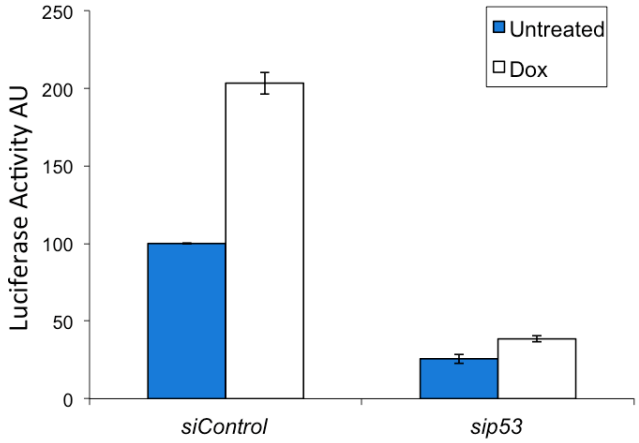


Fig. 2A. Luciferase expression in p21-Fluc/+ MEF lines. Luciferase expression (arbitrary units, AU) in MEF lines 6,7 and 8 was compared under basal condition or in presence of acute-DNA damage causing agent, doxorubicin (Dox, 0.2 µg/ml for 8 hr). Robust reporter activity was detected from one thousand cells in a microwell plate and this activity was induced by doxorubicin (Dox) treatment.

Figure 2

B



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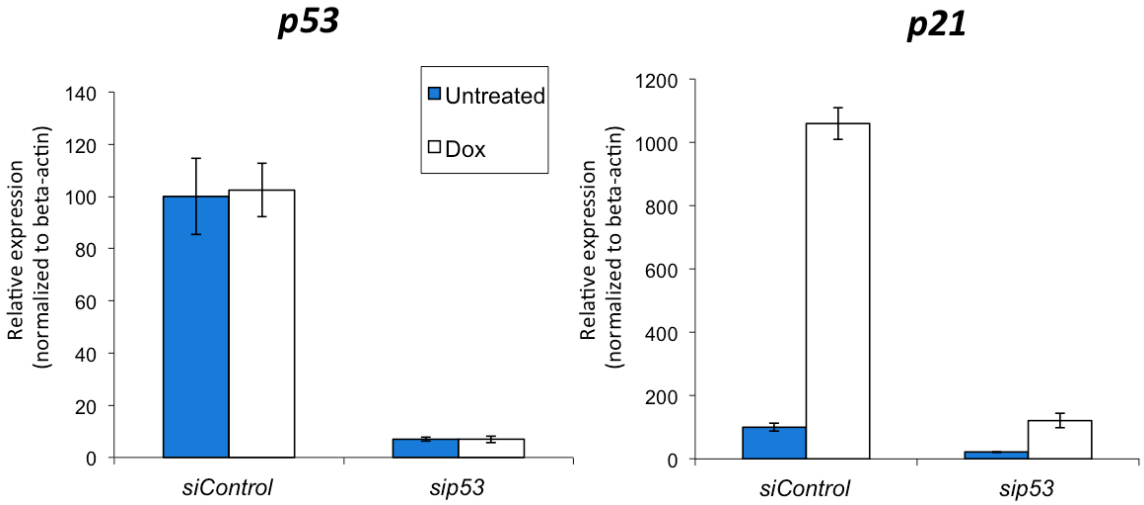

Fig. 2B. Luciferase activity in p21-FLuc MEFs after p53 knock-down in presence or absence of acute-DNA damage. **(C)** qRT-PCR analysis of p21 and p53 in DNA damage-treated p21-Fluc MEFs after p53 knock-down. Graphs indicate averages +/- SD of quantities normalized to β-actin. In both 2B and 2C, cells were treated with 0.2 μg/ml of doxorubicin (DOX) for 8 hours and data is representative of two MEF lines.

Figure 3

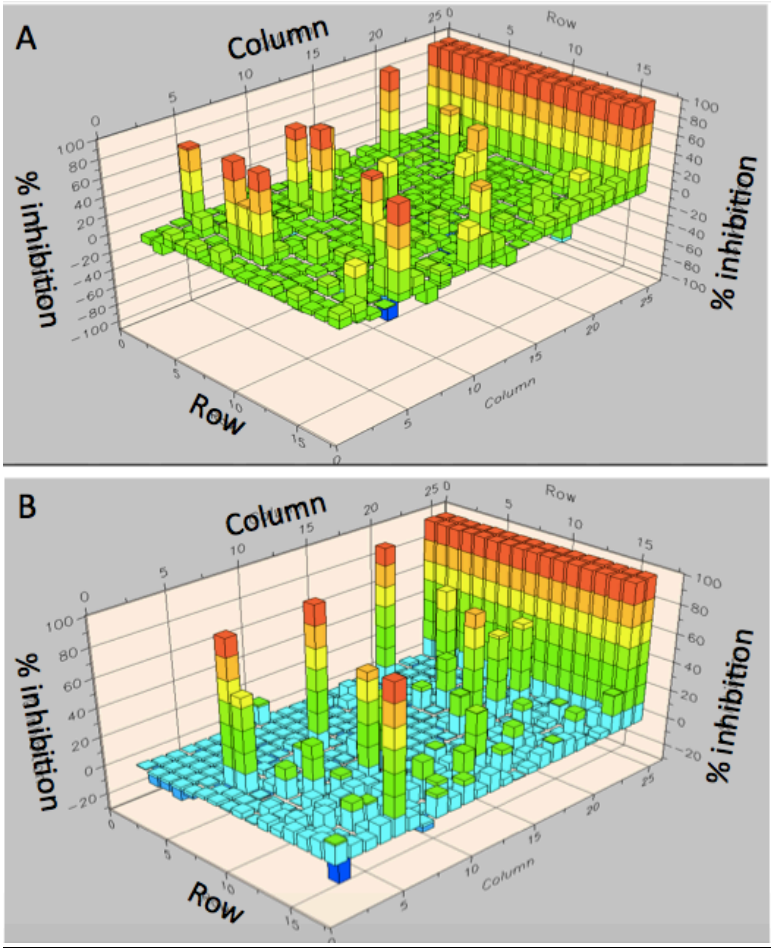

Fig. 3. 3D CityScape bar plot showing percent inhibition of luciferase activity (A) and CellTiter-blue luminescence (B) in a 384-well plate with cultured p21-Firefly Luciferase MEFs treated with compounds from the Microsource Spectrum library.

REPORT OF INVENTIONS AND SUBCONTRACTS

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Leland Stanford Junior University	W81XWH-12-1-0175			a. INTERIM [ ] b. FINAL [X]
b. ADDRESS *(Include ZIP Code)*	**d. AWARD DATE** *(YYYYMMDD)*	**b. ADDRESS** *(Include ZIP Code)*	**d. AWARD DATE** *(YYYYMMDD)*	**4. REPORTING PERIOD** *(YYYYMMDD)*
450 Serra Mall Stanford, CA 94305-2004	20120930			a. FROM 20120930 b. TO 20150929

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
	None							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*	(c) ADDRESS OF EMPLOYER *(Include ZIP Code)*		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION

SECTION III - CERTIFICATION

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I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Fisher, Karen C.	Research Process Manager	Karen Fisher	1/8/16

Award No. W81XWH-12-1-0175

(1) Final SF425, “Federal Financial Report” – submitted by Office of Sponsored Research, Stanford

(2) Final Technical Report – uploaded onto https://ers.amedd.army.mil

(3) Final DD Form 882, “Report of Inventions and Subcontracts” – attached to Final Technical Report

(4) Cumulative listing of only the nonexpendable personal property acquired with award funds for which title has not been vested to the recipient – N/A

(5) “Volunteer Registry Data Sheet,” USAMRDC Form 60R – N/A