Laboratory Exercise

Using Yeast to Determine the Functional Consequences of Mutations in the Human p53 Tumor Suppressor Gene: An Introductory Course-Based Undergraduate Research Experience in Molecular and Cell Biology

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Abstract

The opportunity to engage in scientific research is an important, but often neglected, component of undergraduate training in biology. We describe the curriculum for an innovative, course-based undergraduate research experience (CURE) appropriate for a large, introductory cell and molecular biology laboratory class that leverages students’ high level of interest in cancer. The course is highly collaborative and emphasizes the analysis and interpretation of original scientific data. During the course, students work in teams to characterize a collection of mutations in the human p53 tumor suppressor gene via expression and analysis in yeast. Initially, student pairs use both qualitative and quantitative assays to assess the ability of their p53 mutant to activate expression of reporter genes, and they localize their mutation within the p53 structure. Through facilitated discussion, students suggest possible molecular explanations for the transactivation defects displayed by their p53 mutants and propose experiments to test these hypotheses that they execute during the second part of the course. They use a western blot to determine whether mutant p53 levels are reduced, a DNA-binding assay to test whether recognition of any of three p53 target sequences is compromised, and fluorescence microscopy to assay nuclear localization. Students studying the same p53 mutant periodically convene to discuss and interpret their combined data. The course culminates in a poster session during which students present their findings to peers, instructors, and the greater biosciences community. Based on our experience, we provide recommendations for the development of similar large introductory lab courses.

Keywords: laboratory; curriculum; CURE; p53

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Introduction

The thrill of exploring research questions with unknown answers has often been available only to a select few undergraduates doing individual research apprenticeships with faculty [1]. Recent proposals have called for broadening participation in these experiences to a larger and more diverse population of undergraduates [2–4]. One solution is to embed genuine research experiences within the undergraduate curriculum as formal lab courses [5]. In these course-based undergraduate research experiences (CUREs), students produce original data that address a research problem of interest to scientists outside of the course and may be published in peer-reviewed journals. This distinguishes CUREs from traditional “cookbook” lab courses in which students explore a question with a known answer, and from “inquiry-based activities” in which students explore something that could be novel, but is unlikely to result in publishable material. Additionally, students in CUREs learn and use many of the same scientific skills as students who are engaged in independent research: scientific practice and collaboration, experimental design and iteration, and analysis and interpretation of primary data [5, 6]. Since a research experience early in a student’s academic career is positively correlated with retention in the field [7], a CURE designed for introductory biology students may be a practical way to deliver this experience to a large number of students at a critical point in their development.

Projects must be designed differently for a CURE than for an individual undergraduate working in a faculty mentor’s research lab. Student backgrounds, experiences, interests and motivations vary more widely in a CURE than for students initiating independent research studies, particularly if the CURE is a required, high-enrollment course geared toward a larger, diverse population of early undergraduates [8]. Furthermore, students commit only a few hours at designated times each week to a lab course, compared to longer, flexible hours for students doing independent research. A CURE has a finite time frame that is dictated by the academic calendar, whereas an independent project often spans one or more years. The student-to-mentor ratio in a CURE is significantly higher than the more personal mentoring provided to the average undergraduate independent researcher. Finally, the CURE must be logistically manageable. For example, it is important to consider what resources (personnel, reagents, space) can be devoted to a CURE, how the research project can be organized into discrete units of time, how potentially hundreds of students can work on a project that offers both enough similarities to provide a common curriculum and differences to give students a sense of project ownership, and how instructional guidance can ensure that students are thinking and learning, rather than simply “going through the motions.” Doing this at scale is particularly challenging. Yet, despite these challenges, studies indicate that both types of experiences improve students’ ability to engage in scientific practices and their desire to pursue additional research opportunities (e.g., [9–13]). These findings have prompted national calls for implementation of CUREs at the introductory level [2].

In this article, we describe a curriculum designed and implemented at Stanford University as the first laboratory course for sophomore-level Biology majors and other premedical students. Most of these students enroll concurrently in an introductory biology lecture-based course. The course combines a question of significant biomedical relevance that piques student interest, analysis of mutations in the tumor suppressor gene p53, which is mutated in ~50% of cancerous tumors [14], with a tractable experimental system, the yeast Saccharomyces cerevisiae. Here we present a detailed curriculum with experimental methods and protocols together with sample student results and analyses. We reflect on the learning outcomes for students and provide recommendations for others interested in developing a CURE for large undergraduates classes.

Methods

Course Organization

The Core Molecular Biology Laboratory course meets twice weekly, for separate lecture/discussion and laboratory sessions over 10 weeks, and is offered in the Fall and Winter quarters with a typical enrollment of 60-160 students. During each quarter, students collectively analyze five different p53 missense mutations that have been identified in one or more human tumors, but have not been fully characterized. Over the quarter, students learn and apply basic cell and molecular biology techniques to answer an overarching longitudinal question: What, if any, functional defects are caused by a particular missense mutation in the human p53 tumor suppressor gene? The arc of questions and experiments is summarized in the time line shown in Fig. 1. Note that some questions require several weeks to obtain a final result. Each academic year, five different mutations are examined, allowing the same protocols and experiments to be used to generate novel results.

Each Monday, all students attend a common, 75-min instructor-led lecture/discussion during which background information relevant to course topics and experimental procedures is discussed. Three times each quarter, this period is used for a “Mutant Group” discussion, in which all students studying the same p53 mutant (typically 6-16 student pairs) share data, jointly discuss their findings, and plan future experiments. Comparison of student results allows overall trends as well as outlying data points to be identified. Thus, this activity provides a platform for students to discuss experimental error and variability, and to gain experience with statistical analysis of original data. Furthermore, combining a relatively large number of independent experimental replicates yields a robust data set for each mutant that is suitable for publication.
On one other weekday, students attend a 4-h laboratory section during which they are briefed on key concepts and techniques, perform experiments, score results, and engage in informal discussions. Several sections meet concurrently each day, with 20 students per section split into adjoining rooms, that are jointly supervised by a PhD-level instructor and a graduate student teaching assistant (TA). The students work in pairs, with each of the five pairs in a room investigating a different p53 mutant. This allows students to have a sense of ownership over “their” p53 mutant within each lab room, while also having a pair of students in the adjoining room with which they can compare data. We call these “mini Mutant Groups.”

### Course Assignments and Grading

Each week, students complete both a pre-lab and post-lab assignment. To scaffold the process of thinking as a scientist for students, we developed a method termed QUERY, an acronym for Question, Experiment, Results, and Your interpretation [8]. The QUERY method encourages students to think about the purpose of each experiment within the overarching research project and to consider how their results and interpretations may differ from those of other students. During pre-lab assignments, students are asked to identify the conceptual question posed by an experiment, explain the design of the experiment, including critical controls, and record this information in their lab notebooks. They also read background material relevant to the lab and are tested through multiple-choice auto-graded questions. During post-lab assignments (submitted jointly by research pairs), students report their results, as well as analyze and interpret their data. Post-labs also include conceptual questions, including some that require students to synthesize conclusions from multiple experiments. Students record protocols and experimental results in laboratory notebooks, whose periodic evaluation contributes to their grade. Students’ ability to analyze and interpret data is assessed through three quizzes containing some questions that are similar to those from pre- or post-lab assignments, and others that require students to transfer knowledge to a new scenario or to think about the data in a different way. At the end of the course, students present their findings in both a lab meeting-style talk to peers in their laboratory section and an interactive poster session that is also attended by faculty and members of the biomedical community. Students are required to assess two posters, one from their mutant group and one describing a different mutant, during the poster session. Note that weekly course materials (background information, protocols, laboratory set-up guides, handouts, and post-lab assignments) are included in Supplemental Materials. Sample questions from quizzes and pre-lab assignments are available upon request.

The course is offered only with a pass/no pass option to discourage competition and promote collaboration, which we feel contributes to students learning “how to think like a scientist”. To receive a passing grade, students must earn at least 70% overall, as well as in each course category: pre-lab assignments, post-lab assignments, quizzes, poster, and participation. Typically, 99% of students pass, and the overall class mean is just under 90%.

### Instructional Team

The course is taught by Ph.D.-level lecturers, with the assistance of graduate student teaching assistants (TAs),
and is overseen by two tenure-track faculty members (M.S.C. and T.S.). One of the instructors (D.H.-S.) works full-time during the summer to test new p53 mutant alleles and develop new lab materials, protocols, and instructional support materials. A second instructor (S.M.) oversees course administration. Three staff members prepare and distribute materials for the weekly laboratories: a full-time lab manager (N.B.), a full-time technician, and a part-time lab assistant.

**Experimental Details**

**Yeast Growth Conditions**
Yeast strains are grown on standard Synthetic Complete (SC) growth medium (Sunrise Scientific) which is modified to contain either low (5 µg/mL) or high (200 µg/mL) adenine. Strains containing a LEU2-marked p53-expressing plasmid are grown and maintained on SC medium lacking leucine with high adenine (“SC –Leu”), except for the ADE2 spot assay, when SC-Leu, low adenine medium is used as recommended [15]. Similarly, the yeast strains carrying a URA3-marked p53/sfGFP plasmid are typically grown and maintained on SC medium lacking uracil with high adenine (“SC –Ura”), but are plated on SC –Ura, containing medium (15 µg/mL) adenine for yeast transformations.

**Selection of Mutant p53 Alleles**
Each year, we select five new p53 mutations, typically four located in the DNA-binding domain (DBD) and one in the oligomerization domain (OD). The p53 mutants examined to date display functional defects that range in severity and include alleles that show either temperature-sensitivity or cold-sensitivity (Table 1). Each summer, one of the instructors (DH-S) checks potential p53 mutant alleles to identify those that 1) produce a functional defect that students can observe and 2) display minimal protein degradation to facilitate analysis of functional assay data. Plasmids carrying each p53-mut allele are separately transformed into the “Bax” p53 reporter yeast strain, DH243 (MATa Bax p53-RE::ADE2::ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 Bax p53-RE::lacZ::URA3::ura3-1) and expression of the Bax p53-RE-driven ADE2 gene assessed at 30 and 36°C, by performing the ADE2 Spot Assay as described below. Plasmids carrying p53-mut alleles that show reduced transactivation of the Bax p53-RE-driven ADE2 gene are then separately transformed into the “Con” and “p21: reporter yeast strains (DH241 and DH242, respectively) and tested for transactivation of the Consensus and p21 p53-RE-driven ADE2 genes, respectively, using the ADE2 Spot Assay. The five p53 mutants selected each year display a wide range of severities and temperature-sensitivity to allow for interesting comparisons among student groups studying the different mutants. Extracts from yeast carrying each p53 mutant are then checked by western blot, as described below, to ensure that at least some p53-mut protein is present. Typically, mutations in the DBD produce little or no change in the steady-state level of p53, whereas, mutations

| Allele  | Domain | Phenotype  |
|--------|--------|------------|
| K132Q  | DBD    | Strong     |
| A138P  | DBD    | Strong     |
| V143A  | DBD    | Strong     |
| P152L  | DBD    | Mild, ts   |
| G154V  | DBD    | Moderate, ts |
| T155N  | DBD    | Strong, ts |
| V157F  | DBD    | Moderate, ts |
| R158C  | DBD    | Mild       |
| R158G  | DBD    | Moderate, ts |
| R158L  | DBD    | Moderate, ts |
| R175H  | DBD    | Strong     |
| P177L  | DBD    | Moderate, cs |
| P177R  | DBD    | Moderate    |
| R181H  | DBD    | Mild, cs   |
| H193R  | DBD    | Strong     |
| Y220C  | DBD    | Moderate, ts |
| C238G  | DBD    | Strong     |
| H233D  | DBD    | Mild, cs   |
| I254F  | DBD    | Moderate, ts |
| S241T  | DBD    | Strong, ts |
| G245C  | DBD    | Strong     |
| G245S  | DBD    | Moderate    |
| G266E  | DBD    | Moderate, ts |
| R273H  | DBD    | Strong     |
| E285K  | DBD    | Moderate, ts |
| R273S  | DBD    | Strong     |
| E286A  | DBD    | Moderate, ts |
| R337H  | OD     | None       |
| R337P  | OD     | Moderate, cs |
| R342P  | OD     | Moderate, cs |
| L344P  | OD     | Moderate, cs |
| D352H  | OD     | None       |

Summary of student data on the thirty-two p53 mutations studied to date in the Stanford p53 CURE: Core Molecular Biology Laboratory

| Allele  | Domain | Phenotype  |
|--------|--------|------------|
| K132Q  | DBD    | Strong     |
| A138P  | DBD    | Strong     |
| V143A  | DBD    | Strong     |
| P152L  | DBD    | Mild, ts   |
| G154V  | DBD    | Moderate, ts |
| T155N  | DBD    | Strong, ts |
| V157F  | DBD    | Moderate, ts |
| R158C  | DBD    | Mild       |
| R158G  | DBD    | Moderate, ts |
| R158L  | DBD    | Moderate, ts |
| R175H  | DBD    | Strong     |
| P177L  | DBD    | Moderate, cs |
| P177R  | DBD    | Moderate    |
| R181H  | DBD    | Mild, cs   |
| H193R  | DBD    | Strong     |
| Y220C  | DBD    | Moderate, ts |
| C238G  | DBD    | Strong     |
| H233D  | DBD    | Mild, cs   |
| I254F  | DBD    | Moderate, ts |
| S241T  | DBD    | Strong, ts |
| G245C  | DBD    | Strong     |
| G245S  | DBD    | Moderate    |
| G266E  | DBD    | Moderate, ts |
| R273H  | DBD    | Strong     |
| E285K  | DBD    | Moderate, ts |
| R273S  | DBD    | Strong     |
| E286A  | DBD    | Moderate, ts |
| R337H  | OD     | None       |
| R337P  | OD     | Moderate, cs |
| R342P  | OD     | Moderate, cs |
| L344P  | OD     | Moderate, cs |
| D352H  | OD     | None       |

Summary of student data on the thirty-two p53 mutations studied to date in the Stanford p53 CURE: DBD – DNA-binding domain, OD – oligomerization domain, Strong – significant phenotype observed in Consensus p53-RE reporter strains, Moderate – phenotype observed in p21 and Bax p53-RE reporter strains at one or both temperatures, Mild – phenotype only observed in Bax p53-RE reporter strains, ts – temperature-sensitive (phenotype more pronounced at 36°C than at 30°C), cs – cold-sensitive (phenotype more pronounced at 30°C than at 36°C)
in the OD typically produce a modest (less than two-fold) reduction in p53 level, and a corresponding increase in lower molecular weight degradation products (DH-S, unpublished observation).

We suggest that instructors teaching this course for the first time use the set of p53 mutants we selected for the Fall, 2012 and Winter, 2013 Stanford p53 CURE: G154V (mut1), T155N (mut2), Y220C (mut3), H233D (mut4), and L344P (mut5). In subsequent iterations, we recommend that instructors choose previously untested p53 mutants so that, as in our CURE, the students are able to produce novel, potentially publishable data.

**Qualitative p53 Transactivation Assays**

**ADE2 spot assay.** Derivatives of yeast strains DH241 (Con), DH242 (p21), and DH243 (Bax) that were previously transformed with plasmids carrying p53-wt, p53-mut1-5 or p53-Δ are grown overnight at 30°C in SC –Leu by staff and stored at 4°C for 1-4 days. Each student group is provided with aliquots of nine reporter yeast strains, each of which carries the ADE2 and lacZ reporter genes driven by a particular p53-RE (either Con, p21 or Bax) and expresses a particular allele of p53 (either wt, mut, or Δ). Students dilute each of the nine cultures 1:10 with sterile water. Each partner then uses a multichannel pipetter to spot 5 μL of each strain onto both an SC –Leu and an SC –Leu, low Leu culture. Each partner then uses a multichannel pipetter to spot 5 μL amount of Leu culture 5-fold in SC –Leu medium that has been pre-warmed to either 30°C or 36°C and grown these cultures at the corresponding temperatures for ~5 h. They harvest cells at mid-log phase (OD600~0.7) and pellet the yeast cells by centrifuging at 2,800 xg for 5 min in an Eppendorf 5810R centrifuge. Finally, they freeze yeast cell pellets corresponding to 35 mL of starting culture and store at −80°C until needed.

Each student pair is responsible for preparing cell extracts from pellets of p53-wt, mut and Δ-containing yeast grown at both 30°C and 36°C (six total). Each pellet is resuspended in 240 μL of Extraction Buffer (100 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM DTT and 1X Sigma Protease Inhibitor Cocktail for yeast and fungi) and transferred to a 2 mL microcentrifuge tube containing 0.3 mL of 0.5 mm glass beads (RPI). Each section’s yeast cells are lysed simultaneously in a cold room (4°C) by four cycles of vortexing in a multiphase vortex at top speed for 2 min, followed by a 1 min break. 80 μL of Extraction Buffer containing 4% Igepal CA-630 is added (bringing the final Igepal concentration to 1%), and the solution vortexed briefly. The lysate is then transferred to a fresh tube and centrifuged at 13,000 rpm for 12 min at 4°C in an Eppendorf refrigerated microcentrifuge. The supernatant from each sample is collected and divided into 25 μL aliquots that are immediately frozen on dry ice. The aliquots are then stored at −80°C for future use. A portion of each extract is also subjected to a Bradford assay using the Bio-Rad reagent to determine the corresponding protein concentration using bovine serum albumin (BSA) as a standard.

**Quantitative β-Gal Assay**

Each student pair performs duplicate assays on all six of their yeast extracts (wt, mut and Δ, 30°C and wt, mut and Δ, 36°C). For each sample, 2.5 μL of extract plus Z-buffer [16] to a final volume of 100 μL is placed in a microtiter well. The instructor or TA then starts all reactions by adding 20 μL of ONPG (4 mg/mL) to each well using a micropipette. Production of the yellow-colored product, ONP, by β-gal is monitored by measuring the A420 every min for 30 min using an iMark microplate reader (Bio-Rad).

**Western Blot Analysis of Steady-State Levels of Mutant p53 Proteins**

Each pair of students subjects a 30 μg aliquot of each of their six yeast extracts (wt, mut and Δ, 30°C and wt, mut and Δ, 36°C), as well as Bio-Rad Dual-Color Protein Markers, to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-PROTEAN TGX precast 10% polyacrylamide gel (Bio-Rad) using a Bio-Rad Mini-PROTEAN Tetra System by running at 200 V for 35 min. They then transfer the proteins to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer’s instructions. They stain their nitrocellulose membranes with Ponceau S (0.1% in 5% acetic acid) to confirm similar levels of protein loading and successful protein transfer. They then bisect the membrane just below the 37 kDa marker band and incubate both membrane pieces in a blocking solution consisting of TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, and 0.1% Tween-20) containing 5% nonfat dry milk and 0.01% sodium azide at 4°C until the next week’s lab period.
Students incubate the top membrane piece with the mouse z-p53 monoclonal antibody, DO-1 (EMD Millipore), followed by an HRP-conjugated goat z-mouse 2 antibody (EMD Millipore) to detect p53, whereas they incubate the bottom membrane piece with a rabbit polyclonal antibody to the nuclear protein, Ran (Abcam), which serves as a loading control, followed by an HRP-conjugated goat z-rabbit 2’ antibody (EMD Millipore). Both the 1’ and 2’ antibody for the top piece are diluted 1:10,000, whereas those for the bottom piece are diluted 1:5,000. Immunodetection is performed colorimetrically using a 1-component TMB membrane peroxidase substrate (KPL). Students measure the intensity of the p53 and Ran band in each lane by using ImageJ [17]. They calculate the normalized p53 level in each lane as the ratio of p53 to Ran intensity in that lane. Finally, they calculate the amount of normalized p53-mut protein at both 30°C and 36°C as a percent of the corresponding normalized p53-wt level.

**DNA-Binding Assay**

Students assess the ability of p53 proteins present in their yeast extracts to bind to p53-RE DNA sequences (Con, p21 and Bax), as well as two control DNAs: Random (a random sequence that lacks a p53-RE) and Bgnd (a 5-bp linker sequence). Staff prepare the double-stranded, singly biotinylated DNA oligonucleotides used in the DNA-binding assay by combining pairs of complementary oligonucleotides (each at 10 μM, one carrying a 5’ biotin) in annealing buffer (10 mM Tris, pH 8; 50 mM NaCl; 1 mM EDTA), heating to 95°C for 10 min, and allowing the solution to cool slowly to room temperature. They then dilute the oligonucleotides 10-fold with sterile water to produce a 2 μM double-stranded oligonucleotide stock, that is stored at −20°C. The oligonucleotide pairs used are listed below; the p53-RE sequences are underlined:

- **Con_B-5** (biotin-CTAACGGCGATGTCCGGCGCATGTTCCGGAAAG) and **Con_3** (CTTTCGACAGCTCCGGCGCATGTTCCGGAAAG) and p21_3 (CTTTCAACATGGGACATGGGTCGTGGT1), Bax_B-5 (biotin-CTAACCTCAGCTGGACATGGTGGGAAAG) and Bax_3-2 (CTTTCAGGCTGTTCCTAACTGTGGTGAAG) and Bax_3-2 (CTTTCAGGCTGTTCCTAACTGTGGTGAAG) and Random_3-2 (CTTTCAGGCTGTTCCTAACTGTGGTGAAG) and Random_3-2 (CTTTCAGGCTGTTCCTAACTGTGGTGAAG) and Random_3-2 (CTTTCAGGCTGTTCCTAACTGTGGTGAAG) and Bgnd_5 (biotin-CTAAC) and Bgnd_3 (GTGAAG).

Students use the Epigenetek EpiQuik™ General Protein-DNA Binding Assay Kit (Colorimetric) according to the manufacturer’s directions with the following modifications: Staff wash the Avidin-coated wells three times and then incubate with 50 μL of Binding Buffer (PD2, Epigenetek) containing 40 ng of biotinylated double-stranded oligonucleotide for 1-4 days at 4°C prior to each class. Students remove the biotinylated DNA solution from each well and replace it with 30 μL of protein extract sample containing 25 μg of yeast extract and freshly prepared Extraction Buffer (100 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM DTT and 1X Sigma Protease Inhibitor Cocktail for yeast and fung!) containing 1% Igepal CA-630 (5 μL total) plus 25 μL of Binding Buffer (PD2, Epigenetek). All steps done by the students are performed at room temperature, as recommended by the manufacturer. We have observed that raising the temperature of the p53 DNA-binding step to 30°C or, especially, 36°C dramatically increases the background (DH-S, unpublished observation). For the primary antibody incubation step, DO-1 (EMD Millipore) is diluted 1:500, and the incubation time is shortened to 45 min. The HRP-conjugated goat z-mouse 2 antibody (EMD Millipore) is diluted 1:2,000. Reactions are terminated after 2 min by the addition of Stop Solution. The A_{450} for each well is measured with an iMark microplate reader (Bio-Rad).

Students calculate the level of p53 binding to a particular p53-RE (Consensus, p21 or Bax) or a random DNA sequence lacking a p53-RE (each singly biotinylated and flanked by 5 bp linkers) for each extract by determining the mean A_{450} for wells with that extract and DNA combination and then subtracting, as background, the mean A_{450} of wells that contain the same extract but only the 5 bp biotinylated linker DNA (“Bgnd”). In earlier iterations of the course, we used no DNA as a background control, but its background signal was noticeably higher than that of the Bgnd DNA, possibly due to nonspecific binding of p53 to the avidin-coated wells in the absence of the biotinylated DNAs. Students then calculate the DNA-binding activity of each p53 mutant extract as a percentage of that of the corresponding p53-wt extract.

**Localization of GFP-Tagged Mutant p53 Proteins within Yeast Cells**

**Preparation and verification of yeast strains carrying GFP-tagged p53-mut.** Students transform [16] competent yeast cells prepared by lab staff of strain DH124 (MATa::ADE2::ade2 leu2-3,112 trp1-1 his3-11,15 HTB2::mCherry ura3-1) with DNA of a URA3-marked plasmid carrying their p53-mut/GFP fusion gene and select transformants on SC–Ura, plates containing a medium concentration (15 μg/mL) of adenine. They then use colony PCR to verify that transformants indeed carry the recombinant plasmid with the correct p53-mut/GFP allele. They use the REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturer’s directions to prepare DNA from putative p53-mut/sfGFP-carrying yeast colonies as well as to PCR-amplify p53 fragments from that DNA. Each PCR reaction includes two PCR primers designed to amplify an ~400 bp product from p53. Some of the reactions contain general p53 primers designed to anneal to p53-wt, as well as all p53-mut alleles. The general p53 primer pairs we use depends on the location of the p53 mutant alleles and are either p53_5-4 (ACATAGTGGTGTTTGCGACC) and p53_3-4 (CCTGATTCAATCCTCGGAAAC), or p53_5-12 (GCTGCTCAGATA GCGATGGT) and p53_3-13 (GAGGAGCTGTTGTTGGG).
Other PCR reactions include a “mutant-specific” p53 primer pair designed to amplify an ~400 bp product from the corresponding p53-mut template DNA but not from p53-wt or any other p53-mut. Each mutant-specific p53 primer pair we use includes one primer whose 3’ nucleotide is present in the corresponding p53-mut allele but not in p53-wt or any other p53-mut. Each PCR reaction also includes a pair of control primers designed to amplify a 598 bp product from the yeast URA3 gene: URA3_5-2 (CATGCAAGAAAAGCAAACAAACTTG TG) and URA3_3-2 (GAGACCACATCATCCAGGTCT).
amplification consists of 95°C for 3 min, followed by 34 cycles of 95°C for 30 sec, the optimal annealing temperature for 30 sec, and 72°C for 45 sec, followed by 72°C for 5 min. The optimal annealing temperature is determined empirically and has ranged from 55-64°C. Students analyze their PCR products by subjecting them to electrophoresis on a 0.8% agarose gel containing ethidium bromide and visualizing the DNA bands using a Gel Doc XR (Bio-Rad).

Localization of GFP-tagged p53 proteins in yeast via fluorescence microscopy. The DH124 yeast host strain carries an HTB2-mCherry-spHIS5 allele [18] obtained from Andreas Dončić and Jan Skotheim. This strain expresses mCherry fused to the HTB2 histone subunit, allowing visualization of nuclei by fluorescent microscopy. Staff grow DH124 transformants carrying either p53-wt/GFP, p53-mut/GFP, or GFP alone overnight at 30°C in SC –Ura medium containing the normal level of methionine (150 mg/L). After cells have reached an OD600 of approximately 0.2, the cultures are diluted 6-fold into SC –Ura medium lacking methionine and grown for an additional 3 h at 30°C. The final concentration of methionine during the last 3 h of growth is 25 mg/L, a concentration that induces expression of the p53/GFP gene, which is under the control of the yeast MET25 promoter.

Students view yeast cells with an Evos (Life Technologies) fluorescence microscope using the 40X objective and a 2-4X zoom. Three images of the same field are taken with (1) transmitted light; then (2) the Texas Red Light Cube, to visualize the nuclear-localized mCherry signal; and (3) with the GFP Light Cube, to visualize p53-GFP. Students use ImageJ [17] to merge the mCherry and GFP images. By using the Line tool in ImageJ [17], students determine the maximum nuclear and cytoplasmic signal for five representative cells for each strain (p53-wt/GFP, p53-mut/GFP and GFP alone) and then calculate the mean nuclear:cytoplasmic GFP ratio for each of the three strains.

Results

Course Introduction

During the first two weeks, instructors present an overview of cancer, p53 structure and its role as a tumor suppressor, and the use of yeast as an experimental organism to examine p53 function. Students learn that in response to stress, human p53 activates the expression of target genes that contain p53-binding DNA sequences, i.e. p53-response elements (p53-REs), including p21 and Bax, which promote cell cycle arrest and apoptosis, respectively [19, 20]. Thus, cells expressing mutant p53 that cannot induce transcription of p21 and Bax fail to undergo cell cycle arrest or apoptosis in response to DNA damage, leading to unregulated cell division, additional mutagenesis, and, potentially, tumor formation [20].

Normal (“wild-type”) strains of Saccharomyces cerevisiae do not contain either p53 or p53 target genes. However, previous work showed that the ability of human p53 to express its target genes could be assessed in yeast, providing a rapid, simple and inexpensive method to measure p53 function [15, 21]. Therefore, to measure p53-dependent transcriptional activation, we engineered a series of yeast strains that express both p53 and two p53-dependent reporter genes (Fig. 2A). These p53 reporter strains each carry a single, constitutively expressed, plasmid-borne allele of human p53: either wild-type (p53-wt), a mutant carrying a single, missense mutation (p53-mut1, 2, 3, 4 or 5), or an internally deleted p53 gene that produces a truncated, nonfunctional p53 protein (p53-D). In addition, each strain carries two p53 reporter genes, the yeast ADE2 gene and the E. coli lacZ gene, at separate genomic loci, but both under the control of the same p53 response element (p53-RE). Three p53-REs are used: Consensus (an artificial sequence representing a “best match” for all p53-REs [22]), the p53-RE from the p21 gene promoter, and the p53-RE from the Bax gene promoter. The affinity of p53 for these p53-REs varies from high (Consensus) to intermediate (p21) to low (Bax); thus, together this set of yeast strains allows the transactivation ability of each p53 mutant to be assessed semi-quantitatively.

Initial Experiments

QUESTION: Does the p53 Mutant Exhibit a Transactivation Defect?

Qualitative assays to measure p53 mutant function: In the first two lab sessions, students carry out in vivo assays to investigate the transactivation ability of their mutant p53. These experiments require little technical expertise and are surprisingly robust and reproducible. These sessions introduce students to experimental design; they learn the value of positive and negative controls, and of employing multiple assays, i.e. two different reporter genes (lacZ and ADE2) and three different p53-REs, to measure a wide range of p53 function.

In the first lab session, students learn how to use micropipettes to measure small volumes of liquid, make serial dilutions, spot cultures of yeast on solid medium, and use light microscopy to visualize yeast cells. In the second week, students evaluate the growth and color of yeast cultures they inoculated in week 1 (ADE2 spot assay), and conduct a filter-lift assay for β-galactosidase activity. These assays are carried out as follows:

Experiment #1: ADE2 spot assay. The yeast ADE2 gene encodes the AIR carboxylase enzyme, which converts P-ribosylamino imidazole (AIR) into P-ribosylamino imidazolcarboxylate (CAIR) in the adenine biosynthetic pathway [23]. Wild-type yeast colonies appear white in color. However, in cells lacking the ADE2 gene product, AIR accumulates, and is converted to a product that causes yeast colonies to be red [23], with the degree of coloration being proportional to the reduction in ADE2 transcription; this effect is enhanced on growth medium with a reduced...
adenine concentration. In the reporter strains designed for this course, ADE2 transcription is controlled by a p53-RE, and thus requires functional p53. Hence, the p53-wt spots are white, pink or red, depending on the ability of the mutant protein to bind to and promote expression of each p53-RE-driven ADE2 gene (Fig. 2B, left panel). Students perform the ADE2 spot assay at two different temperatures: 30°C (ideal yeast growth temperature) and 36°C (just below mammalian body temperature) to determine if the p53 mutant displays a temperature-dependent defect in transactivation (Table 1, Fig. 2B, left panel). To allow direct comparison of data from different student pairs, a standardized scoring scheme is used: the p53-mut spot is scored as “3” (full transactivation) if it is white as the corresponding p53-wt strain (positive control), “1” (no transactivation) if it is as red as the corresponding p53-Δ strain (negative control), and “2” (reduced transactivation) if its color is intermediate.

Experiment #2: β-gal filter lift assay. The E. coli lacZ gene encodes β-galactosidase (β-gal), an enzyme that cleaves lactose, a disaccharide, into the monosaccharides glucose and galactose. Each of the yeast strains carries a lacZ gene that is controlled by the same p53-RE that controls expression of ADE2. Students spot their p53 reporter yeast strains onto solid growth medium. Following growth at either 30 or 36°C, students transfer and lyse the yeast cells on filter paper replicas, which are then incubated with the β-gal synthetic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). When cleaved by β-gal, X-gal yields an insoluble, blue product, 5,5’-dibromo-4,4’-dichloro-indigo [24, 25]. Since β-gal production requires functional p53, p53-wt spots are typically dark blue, and the p53-Δ spots are white. Students score the level of p53-mut-mediated transactivation of each p53-RE/lacZ reporter as “3” (full transactivation) if it is as blue as the p53-wt spot, “1” (no transactivation) if it is as white as the p53-Δ spot, and “2” (reduced transactivation) if its color is intermediate (Fig. 2B, right panel).

Interpreting the Qualitative Transactivation Assays

The ADE2 and lacZ assays provide complementary information about p53-mediated gene expression: Activation of lacZ produces a readily observed blue color and, conversely, reduced activation of ADE2 produces a readily observed red color. Thus, the p53-RE/lacZ reporter better discerns low levels of transactivation (just above those of p53-Δ), and the p53-RE/ADE2 reporter better discerns slightly reduced transactivation (just below that of p53-wt). Students determine overall function of their p53 mutant with respect to the various p53-REs and two growth temperatures by averaging the scores for the two assays into a mean “transactivation score” and combine their data with others’ during the Mutant Group discussion (see below).

QUESTION: Where Is the p53 Mutation Located?

p53 structure and function: In the Week 2 lecture, students learn that p53 is a tetramer, and that each p53 monomer contains an N-terminal transactivation domain (TAD), a large, central DNA-binding domain (DBD), and a C-terminal oligomerization domain (OD) [14]. Students learn that p53 must tetramerize via the OD to effectively bind to p53-RE DNA because monomers and dimers have markedly lower DNA-binding affinity [26]. In their pre-lab exercise, students explore the expected consequences of mutations that completely abolish the function of each domain.

During the lab session, students analyze the DNA sequence of wild-type human p53 using BLASTn, ORF-finder and BLASTp to determine the protein sequence and identify related proteins. The pre-lab discussion and student tutorial reinforce concepts from the introductory biology lecture class, such as the central dogma (DNA—RNA—Protein). Students also use Jmol [27], a free, online program, to
examine the structure of a wild-type p53 DBD bound to DNA, and highlight the position of a particular amino acid that directly contacts the DNA. In the post-lab exercise, students use these same online resources to identify the specific nucleotide and amino acid change produced by the p53 mutation they are studying, and highlight the position of the mutated amino acid within the 3D structure of p53’s DBD or OD (Fig. 3B). The majority of p53 mutations used in the class are in the DBD, as this domain is most frequently mutated in tumors (Fig. 3A). To broaden class discussions, we have also included mutations in the OD but not the TAD, because single, tumor-associated missense mutations in the TAD do not appear to significantly reduce transcriptional activation [28].

**QUESTION: What is the Extent of the p53 Mutant’s Transactivation Defect?**

Quantitative assay to measure p53 mutant function: In Weeks 3 and 4 students quantitatively determine levels of p53-dependent β-galactosidase expression in the p53 reporter yeast strains. In Week 3 students prepare yeast cell extracts and measure their protein concentration. In Week 4 they carry out kinetic β-galactosidase assays and transform their primary data into normalized enzyme activities. This experiment demonstrates to students the value of a quantitative versus qualitative (β-gal filter lift) assay. Hands-on manipulation of their data allows them to grapple with data reliability and reproducibility, including using statistical tests to determine significance. Overall, this part of the course builds skills in quantitative analysis.

Preparation of protein extracts and determination of protein concentration (Week 3): Students prepare total protein extracts from their yeast strains via glass bead lysis of frozen pellets of p53-wt, mut and Δ strains grown at 30°C and 36°C (six strains per student pair). Due to time constraints, students analyze only strains that contain the Con p53-RE-driven reporter genes. Next, students use a Bradford assay, which relies on production of a blue-colored product that is monitored by A$_{595}$ [29], to determine the relative protein concentrations of their yeast extracts by comparison to a standard curve. Students are required to accurately assess the relative protein concentrations of their yeast extracts, that will be used for quantitative β-gal assays, as well as other future experiments. Hence, the standard curve must be a good fit for the data (R$^2 > 0.95$), and all data points for the yeast extract samples must fall within the standard curve. If not, students are required to repeat the Bradford assay.

Quantitative β-gal kinetic assay (Week 4 lab): Students use a plate reader to perform a kinetic assay for β-galactosidase activity on their six yeast extracts (wt/30, mut/30, Δ/30, wt/36, mut/36, Δ/36). Students first pipette equal amounts of total protein from all extracts (including duplicates for all samples) into wells of a microtiter plate. Then we add the substrate, ortho-Nitrophenyl-β-galactoside (ONPG), which is cleaved by β-gal to release ONP, a soluble yellow product that is monitored at A$_{415}$. Students measure the A$_{415}$ every minute for 30 min in a plate reader, and calculate the reaction rate (the slope of the initial linear phase of the curve) for each sample. The reaction rate reflects the number of molecules of β-gal present, which reflects the extent of p53-dependent activation of the Con p53-RE-driven lacZ gene. Because the amount of β-gal also increases as a function of total protein content, students calculate the normalized β-gal activity of each extract (in A$_{415}$/min/µg protein) by dividing the reaction rate by the protein content of that sample. Students then compare β-gal activities of their 30°C and 36°C p53-mut extracts to the corresponding 30°C or 36°C p53-wt extract. This quantitative assay allows for more fine-tuned determination of p53 mutant function.

**Mid-Term Reflection**

Half-way through the term, students convene in Mutant Groups to collectively analyze the transactivation data they collected during the initial portion of the course, consider...
hypotheses to explain their p53 mutant’s transactivation defect, and propose experiments for the remainder of the course. Students meet as Mutant Groups to discuss, analyze and interpret their data collectively in Weeks 4 and 6. Although an instructor facilitates the discussion, students are encouraged to take the lead in interpreting their data. In Weeks 4 and 6, students meet in mini Mutant Groups during the lab period to design future experiments. Student handouts for all of these meetings are included among the Supplemental Materials.

Data Analysis

During the Week 4 Mutant Group, students analyze their qualitative transactivation assay data. After noting the location of their mutation, all student data from qualitative transactivation assays is averaged to calculate mean transactivation scores for each p53 reporter strain and growth condition. Students determine if there are valid reasons for excluding any outlying data points from their calculations. This yields a semi-quantitative measure of p53 mutant transactivation ability that is remarkably robust, and allows students to make confident conclusions about the function of their mutant (Figs. 2C and 2D). Student Group conclusions for each mutant under study are discussed in the following lab sessions, allowing students to appreciate that missense alleles differ in severity depending on the position mutated and degree of similarity between the wild-type and substituted amino acids. Students also use online databases to identify published information about the mutation under study, particularly the number and types of tumors in which it has been observed.

In the Week 6 Mutant Group, students combine results of their quantitative transactivation assays to calculate the mean relative β-gal activity of their p53-mutant at both 30°C and 36°C (Fig. 2D) and discuss statistical analysis, data reliability and reproducibility. Students learn that multiple experimental replicates are needed to produce reliable, statistically significant data. The quantitative β-gal assay typically reveals a more dramatic reduction in p53-mut transcriptional activation than observed in the qualitative assays due to its increased precision. These experiments also dramatically illustrate the importance of accurate pipetting for data reproducibility.

Generating Hypotheses and Proposing Future Experiments

After students ascertain that their p53 mutants display reduced p53-dependent gene expression when expressed in yeast cells, they are asked to probe the molecular defects that may underlie their observations. The instructor begins this discussion during the Week 4 laboratory period by describing the steps required for p53 to activate a target gene in either yeast or human cells (p53 cycle, Fig. 4). Students are then asked to identify specific processes that, if impaired, could yield an observed transactivation defect, and propose possible experimental tests of these ideas. This discussion occurs within each four-person team studying the same mutant, with as little contribution as possible from the instructor and/or TA. Students typically identify five possibilities: (1) The mutation causes mis-folding and subsequent degradation of p53; (2) The mutation impairs oligomerization of p53; (3) The mutation impairs localization of p53 to the nucleus, where transcription occurs; (4) The mutation impairs p53 binding to p53-RE DNA sequences; or (5) The mutation impairs recruitment of the RNA polymerase II transcriptional machinery by p53 to initiate transcription of p53 target genes. To support the third possibility, the instructor further expands upon roles of nuclear import vs. export in localization, noting that oligomerization masks the p53 nuclear export sequence (NES), located within the OD, thus contributing to nuclear retention of the p53 tetramer [30]. Overall, the goal of these discussions is to emphasize that students’ knowledge is limited to an observed impairment of p53-dependent gene expression in yeast cells, which could result from one or more defects in distinct, underlying molecular processes. Ultimately, students are guided toward testing three hypotheses experimentally for the remainder of the course:

1. Is the level of p53-mut protein lower than that of p53-wt protein?
   Experimental technique employed: Western Blot.
2. Does p53-mut protein bind p53-RE DNA as well as p53-wt protein?
   Experimental technique employed: DNA-binding assay.
3. Does the p53-mut protein localize to the nucleus to the same extent as p53-wt protein?

Experimental technique employed: Fluorescence microscopy of GFP-tagged p53-wt and -mut

Note: Students working with p53 mutations in the OD often propose a defect in oligomerization as a fourth hypothesis but are guided to acknowledge that this defect should also reduce binding to p53-RE DNA and nuclear localization, both of which will be examined.

Final Experiments: Answering Mechanism Questions

QUESTION 1: Is the Level of p53 Mutant Protein Reduced?

Western blot to compare levels of p53 wt and mut protein in yeast extracts: This experiment is divided between successive lab sessions, with students carrying out SDS-PAGE and transfer of their samples to membranes in Week 5, and immunodetection in Week 6. The experimental design includes positive and negative controls and introduces the concept of a loading control. In Week 5, students subject separate aliquots of the six yeast extracts they analyzed in the quantitative β-gal assay (wt, mut, and Δ extracts of cells grown at 30 and 36°C) to SDS-polyacrylamide gel electrophoresis and electrotransfer to a nitrocellulose membrane, followed by incubation with the protein stain, Ponceau S, to confirm equal loading of each sample and uniform transfer of proteins to the membrane, and storage in blocking solution at 4°C. In Week 6, students use different primary antisera to detect p53 (~53 kDa) and Ran (~25 kDa), an abundant nuclear protein used as a loading control. Therefore, students bisect their membranes just below the 37 kDa marker and incubate each portion with the appropriate antiserum followed by washes, incubation with horseradish peroxidase (HRP)-conjugated secondary antisera and ultimately, tetramethyl benzidine (TMB), an HRP substrate, to visualize the p53 and Ran protein bands colorimetrically. In the post-lab exercise, students compare the amount of p53-wt and mut in their extracts at both 30°C and 36°C by measuring band intensities with ImageJ and calculating the ratio of full-length p53 to Ran intensities for each of their samples.

QUESTION 2: Does the Mutant p53 Protein Display Reduced DNA-Binding?

Comparing the amount of binding to p53 RE-containing DNA by yeast extracts containing either p53-wt or mut: This colorimetric DNA-binding assay is introduced conceptually during a pre-lab discussion in Week 6, and carried out by students in Week 7. In this experiment, students continue to build skills in quantitative analysis and experimental design: student choice dictates 50% of the samples that are analyzed, and students consider how to design a negative control. The assay is carried out in wells of a microtiter plate that are coated with avidin, a protein with exceptionally high affinity for the small molecule, biotin [31]. Biotinylated p53-RE-containing DNA oligonucleotides are first bound to the wells, which are subsequently incubated with p53-containing yeast extracts and analyzed to determine the amount of p53 bound to each well via immunodetection with anti-p53 (primary) and HRP-conjugated (secondary) antisera followed by
colorimetric detection and quantification in a plate reader (Fig. 6A).

**Student-initiated Experimental design:** During downtime in the Week 6 lab, students break into 4-person mini Mutant Groups to plan their DNA-binding assay. Each student pair is given four strips of eight avidin-coated wells, to analyze a total of 32 samples. For 16 of these, the entire class is instructed to analyze eight conditions in duplicate: incubation of yeast extracts containing p53-wt or mut and grown at either 30 or 36°C, and in wells coated with either Consensus p53-RE or a short linker DNA (“Bgnd”). Students determine the components of the remaining 16 wells, but are instructed to test eight conditions in duplicate, and are reminded that each test sample must have a positive and negative control. Students choose from their existing samples, i.e. p53-wt, mut or Δ extracts of yeast grown at 30 or 36°C, and three possible biotinylated DNA oligonucleotides, containing the p21 or Bax p53-RE or a random DNA sequence that does not contain a p53-RE. These choices also allow students to consider different types of negative controls for the experiment: Use of p53-Δ extract controls for any non-p53-derived background signal, whereas incubation of p53-wt and mut extracts in wells coated with DNA that lacks a p53-RE controls for any non-sequence-specific DNA binding activity. Furthermore, each group of students working on the same mutant discusses whether to test identical experimental conditions, allowing more replicates, or more conditions with fewer replicates per condition. In their post-lab exercise, students calculate the background-subtracted DNA-binding activities of their p53-mut extracts relative to the corresponding p53-wt extract as well as relative to the p53-wt extract with the Consensus p53-RE DNA.

**QUESTION 3: Does the Mutant p53 Protein Show Reduced Nuclear Localization?**

**Determination of GFP-tagged p53-wt and p53-mut localization using fluorescence microscopy:** During Weeks 5-8, students use GFP-tagged p53 alleles to quantify levels of p53-wt and p53-mut in the nucleus. In Week 5, students transform a *ura3* yeast host strain containing Consensus p53-RE-driven *ADE2* with *URA3*-marked plasmids carrying either p53-mut/GFP, p53-wt/GFP or GFP alone (Fig. 7A). In Weeks 6 and 7 students isolate p53-mut/GFP transformants that are white in color, indicating that the p53-GFP fusion is functional, and confirm via colony PCR, performed with general p53 or p53-mut-specific primers that cells contain the correct p53-mut/GFP gene. These exercises expose students to a variety of useful molecular experimental techniques, including yeast transformation and colony PCR, and allow them to practice their pipetting and basic laboratory skills.

**Figure 7**

Examining nuclear localization by mutant p53. (A) Schematic diagram of the yeast strain used to monitor the subcellular location of p53 proteins. The strain expresses a p53/GFP fusion gene from an inducible MET25 promoter (depicted by the blue rectangle) contained within a URA3-marked plasmid. The strain carries a consensus p53-RE (represented by four black diamonds) that controls expression of the downstream ADE2 gene; students use expression of this gene to confirm that the p53/GFP fusion protein is functional, i.e. by selecting white colonies on low-adenine growth medium. The strain also expresses a nucleolar histone protein fusion, Htb2-mCherry, which fluoresces orange/red under ultraviolet light. (B) Student fluorescence microscopy data. Expression of GFP-tagged p53-wt (top, positive control), GFP-tagged p53-G266E (middle), or GFP alone (bottom, negative control) was induced by growth in low methionine medium for 3 h, and visualized using an Evos fluorescent microscope. Yeast cells are shown under bright field (left column). Nuclei are visualized by red fluorescence (Htb2-mCherry, second column) and p53/GFP by green fluorescence (third column); these two images are merged in the fourth column. Nuclear enrichment of p53-G266E/GFP is distinguishable from p53-wt/GFP both by visual inspection, and as quantified using the Line tool in ImageJ [17]; the “mut3 Group” determined the nuclear:cytoplasmic ratio of GFP fluorescence as 2.1 ± 0.4 for p53-G266E compared to 2.3 ± 0.9 for p53-wt. Data were produced by Jeffrey Kwong, who took the Stanford p53 CURE in Winter, 2014. [Color figure can be viewed at wileyonlinelibrary.com]
biology techniques, i.e. transformation, PCR, and agarose gel electrophoresis, but could be omitted to shorten the laboratory exercises.

In Week 8, students use fluorescence microscopy to quantify and compare the extent of p53-wt/GFP (positive control), p53-mut/GFP and GFP (negative control) localization to the nucleus. The host strain carries a tagged histone, mCherry-HTB2, which fluoresces orange-red when excited that is used to visualize nuclei within their p53/GFP-expressing yeast cells. In their post-lab, students use ImageJ [17] to overlay GFP and mCherry images and determine whether p53-mut/GFP and p53-wt/GFP exhibit a similar degree of nuclear localization. This is analyzed qualitatively (by eye) and quantitatively, using the line tool in ImageJ to determine ratio of nuclear:cytoplasmic GFP signal for the p53-mut/GFP, p53-wt/GFP and GFP expressing yeast strains (Fig. 7B).

**BRINGING IT ALL TOGETHER**

**Final Mutant Group Discussion and Journal Club**

The Week 9 lecture period is devoted entirely to a Mutant Group Discussion in which students compile their data for the western blot, DNA-binding assay, and fluorescence microscopy experiments and discuss their overall conclusions concerning molecular characterization of the transactivation defect observed for their p53 mutant. Students first examine their compiled western blot data and calculate the mean p53-mut level (as a % of p53-wt) at both temperatures and consider whether reduced levels of p53 mut protein can account for the transactivation defect observed. Typically, they conclude that a reduced level of p53-mut protein cannot fully account for the observed transactivation defect.

Next, they examine their compiled DNA-binding data and calculate the mean DNA-binding activities of p53 mut vs. wt extracts (Fig. 6B). Students consider whether a reduction in binding to p53-RE containing DNA observed for p53-mut versus wt yeast extracts in this experiment likely explains the reduced transactivation activity documented in Weeks 1-4. Mutants with mild in vivo transactivation defects often display more dramatic deficits in the DNA-binding assay, allowing students to discuss differences between in vivo and in vitro measurements.

Finally, students calculate the mean nuclear:cytoplasmic ratio observed for their p53-mut/GFP and control yeast strains, and determine if reduced localization of p53-mut/ GFP to the nucleus can explain its observed transactivation defect. By combining their data, students are often able to see trends—i.e. slightly reduced or even increased nuclear localization of p53-mut/GFP—that is not always evident in a single data-set.

During the Week 9 lab period, the instructor or TA leads the students in a discussion of a paper from the scientific literature [32] in which investigators use similar methods to identify a molecule that reactivates mutant p53 and is currently in clinical trials as an adjuvant to chemotherapy [33].

**Final Presentations**

During the Week 10 lab session, each section of 20 students participates in a “lab meeting,” during which each 4-person mini Mutant Group delivers a 10-min presentation on their results and conclusions to the group. A modest participation incentive encourages students in the audience to ask questions after each presentation. Questions typically address discrepancies in the data (e.g. why a mutant appears temperature-sensitive in the transactivation assays, but not in the DNA-binding assay) or variation between different mutant phenotypes, as well as concerns about data interpretation, requests for more background information about a particular p53 mutant, or details for suggested future experiments.

As a culmination of their work in the course, each student pair prepares a poster highlighting their results and conclusions about their p53 mutant’s transactivation defect. Students from every section of the course present their posters during a common poster session, which is open to members of the Stanford community. During the poster session, each student is required to complete a questionnaire on two posters: one on the same p53 mutant s/he studied during the course and one on a different p53 mutant. As a consequence, the poster session is a highly interactive event where students gain further insight into their p53 mutant, as well as other p53 mutants studied in the course.

**Discussion**

This paper describes the curriculum for a CURE that has been offered 11 times with minor annual modifications, and with proven positive outcomes on student learning [8]. It is relatively straightforward to design a CURE based on a faculty research project, due to the availability of knowledge, infrastructure and trained personnel. Our CURE is distinct in that it is not directly related to a faculty member’s research program, although it is informed by the experience of the faculty and staff who designed it. This independence from dedicated local research effort is a strength in that it has made for a course that is more readily transportable to other institutions and settings. The course is designed so that, in a 10-week quarter, students progress from knowing little about the p53 tumor suppressor protein, the specific focus of the course, or the experimental process, to reporting extensive characterization of a mutant p53 through well-defined and controlled experiments. Since the course has focused on characterizing p53 mutants of interest to the research community, we anticipate that we will publish a research article based on our students’ research findings within the next few years after acquiring, through repetition, data of sufficient quality. Instructors offering a related course at other institutions could
also potentially publish their students’ analyses of other, previously uncharacterized, p53 mutants. We discuss here recommendations for implementing such a course, lessons learned in creating and offering this course, and ways in which it could be adapted to other situations, for example, a semester-long course, or a course with substantially fewer available resources.

Recommendations for Implementing a Stand-Alone CURE Lab Course

Based on our experiences developing and teaching this CURE, we have identified eight key features of a successful research-based course that functions independently from a faculty research project. These are indicated below, with specific examples of how each feature was achieved in our case study.

Connection to a Real-World Problem

- Students are more easily engaged with problems of clear significance to the human condition.
- Based on our high pre-med population, we choose to focus on a protein important in cancer.

Required Introductory Course

- Making the course a major requirement ensures that all students understand the experimental underpinnings of biology.
- This course is designed for sophomore-level students and builds on concepts introduced in lecture courses.

A Longitudinal Research Question Common to all Students

- By having all students work on the same research question with the same set of experiments, the course is both logistically feasible, and intellectually satisfying.
- All students investigate the functional defects of p53 mutants using the same series of molecular biology experiments.

Student Ownership Through Variation in Elements of the Research Project

- Incorporating variation into the research project, both in the particular mutant allele of p53 studied, and aspects of the experiments performed, increases students’ sense of ownership and thus, engagement.
- Student pairs work with one mutant allele of p53 for the entire quarter, with different student pairs in the same room investigating different mutants. Students may choose different conditions for the DNA-binding assay, based on determined properties of their mutant.

Collaboration Across Laboratory Sections, Sharing Data to Build Larger Datasets

- The large student population in this course, often a detriment in lab courses, is leveraged by having student pairs combine data to achieve multiple replicates of the same experiment. This allows students to draw statistically significant conclusions.
- Student pairs studying the same p53 mutant convene for Mutant Group Discussions in which they compare data, generate conclusions, assess their significance, pose hypotheses, and plan future experiments.

Community of Collaborators Rather Than Competitors

- Create a congenial, community atmosphere to the lab setting, with an emphasis on the experimental process rather than obtaining a predetermined “right answer.”
- To encourage the sharing of data and ideas and reduce their natural competitiveness, students take the course pass/fail rather than for a letter grade.

Instructors Who Think as Scientists

- Instructors with experience in scientific research are best able to teach a high level of scientific thinking.
- We have Ph.D. level instructors teaching each section of the course, with graduate students serving as teaching assistants.

Course Assessments That Are Authentic to the Research Process

- Organize assessments that parallel real-life experiences in science, such as lab meetings, oral discussions and poster presentations.
- Student pairs give 10-min oral presentations in an informal style typical of a lab meeting presentation and engage in Q & A throughout the presentation. For the culminating assessment, students present a poster in a common poster session open to members of the academic community.

We have, through several iterations, designed the skills component of the course to achieve accuracy and reproducibility of the experiments by the majority of students given their varying initial laboratory skills and attention to detail. This is a challenge, since the majority of the experiments are dependent on accurate performance of molecular biology techniques, some of which are difficult for introductory students to master. Accordingly, we have incorporated opportunities for students to practice these skills, as well as to share data so they can be more confident in the conclusions of a particular mutant.
Lessons Learned

Through 11 iterations of the course, we have revised the curriculum based on both student and instructor feedback. Below are some important lessons that we have learned.

Course logistics:

1. It is helpful to have a full-time team-member who can work during periods when the course is not offered to prepare laboratory materials (new yeast strains and plasmids), introduce new protocols or optimize current ones, and update written materials. It is also helpful to have a technical support person(s) to prepare and set-up weekly lab materials.

2. Having students work as pairs on course assignments (post-lab assignments and final poster) leads to improved performance on those assignments. Assessing individual understanding can be done with quizzes that are completed independently.

3. Requiring that students submit a spreadsheet with their data analysis allows TAs to more readily assess quantitative analyses in conjunction with instructor-provided templates.

4. Color coding all student materials (racks, tubes, tape, etc.) by mutant (i.e. mut1 = orange, mut2 = blue, mut3 = pink, mut4 = yellow, mut5 = green) facilitates their distribution and organization.

5. Mistakes (by both staff and students) will happen, but it is often possible to make these into good learning opportunities.

Specific experimental logistics:

1. We have found that p53 proteins (particularly the mutant versions) are highly susceptible to proteolysis. Hence, it is essential that once students lyse their yeast cells, they keep their samples at 4°C and work efficiently to minimize the time before they aliquot and freeze their protein extracts.

2. The DNA-binding assay is prone to high variability. We have found that the following tips help, but do not eliminate this variability: (A) do not leave wash buffer in wells, and do not to allow wells to dry, as both have the potential to increase background; (B) do not scrape the bottom of the wells with pipet tips as this can remove the avidin coating holding the biotinylated DNAs to the well; (C) the assay must be done at room temperature as the background increases dramatically when performed at 30°C and (especially) 36°C; and (D) when there is little or no specific DNA binding, background subtraction will sometimes yield “negative” binding activity; students should be reminded of this possibility.

3. In Week 3, Bradford Assay data should be checked by instructors to confirm that they fall within the linear range of the standard curve; students should repeat the analysis if the $R^2 < 0.95$ or one or more data points are not within the linear range of the standard curve.

4. The major source of experimental error throughout the course has been inaccurate pipetting. We have found it helpful to emphasize proper pipettor technique and establish quantitative criteria for duplicates, i.e. they should not differ by >50%, that students must meet for their data to be included in further analysis.

We teach this course in a 10-week quarter format. For instructors who are considering this curriculum for a semester-long lab course, additional lab experiments could be added. For example, students could make their own GFP-tagged p53 mutant constructs, using homologous recombination in vivo, and confirm by sequence analysis; students studying the putative oligomerization mutants could assess oligomerization state using native gel electrophoresis; students could test more parameters of DNA-binding, such as other DNA elements or extracts; and all students could have one or two periods to repeat an experiment of choice. Having students repeat experiments might be the most productive use of the extra weeks to help solidify student understanding of the inherent “messiness” of data and the need for multiple replicates; it would also increase the probability of getting publication-quality data.

Limitations

This CURE was implemented at an institution with, mostly, highly motivated students and significant resources relative to those that might be available at many other institutions. This provided several benefits – for example, having a team of Ph.D.-level instructors teaching the course, and well-trained graduate students serving as teaching assistants. However, we believe that other institutions with different student populations and/or fewer resources could effectively implement this CURE, perhaps removing some of the more expensive experiments (e.g. DNA-binding assay) or experiments that require more expensive instrumentation (e.g. fluorescence microscopy). Preparing the reagents for DNA-binding assays in-house [34] could also lead to significant cost-savings. Some of the most important elements of the course have to do with structure, rather than specific experiments, such as the mutant group discussions that bring together “independent researcher” students to discuss and assess their data on a common mutant. These elements can be implemented in the context of any lab course in which students study related variants of a particular problem. Portions of the course can also be taught, particularly when time and/or experimental resources are limiting. For example, during two-week intensive cell biology workshops at the University of Ghana (taught by M.C. and T.S.), students with limited background in cell and molecular biology carried out structural, gene expression, western blot and fluorescence microscopy analyses of yeast strains that expressed p53-wt, p53-mutant (R175H or Y220C) or p53-Δ. Over the course of three afternoons, these exercises
effectively introduced students to the molecular basis of cancer and gave them hands-on experience with experimental design and data analysis.

Conclusions
We present this curriculum for instructors who would like to replicate this course at their own institution, and as a model for instructors who are interested in developing their own, related, CUREs. We are eager to share resources, reagents, and course materials to help instructors transition their traditional introductory lab courses into CUREs. We hope that this course can serve as an example that can help others reform their lab courses to integrate teaching and research so that all undergraduates can experience the excitement of discovery through scientific research.

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References
[1] Laursen SL, Hunter A-B, Seymour E, Thiry H, Melton G (2010) Undergraduate Research in the Sciences: Engaging Students in Real Science. San Francisco: Jossey-Bass.
[2] Brewer CA, Smith DP (2011) Vision and Change in Undergraduate Biology Education: A Call to Action. Washington, DC: American Association for the Advancement of Science.
[3] Bangera, G., and Brownell, S. E. (2014) Course-based undergraduate research experiences can make scientific research more inclusive. CBE Life Sci. Educ. 13, 602–606.
[4] Olson, S., and Riordan, D. G. (2012) Engage to Excel: Producing One Million Additional College Graduates with Degrees in Science, Engineering and Mathematics. Washington, DC: President’s Council of Advisors on Science and Technology.
[5] Auchincloss, L. C., Laursen, S. L., Branchaw, J. L., Eagan, K., Graham, M., et al. (2014) Assessment of course-based undergraduate research experiences: A meeting report. CBE Life Sci. Educ. 13, 29–40.
[6] Brownell, S. E., Kloser, M. J., Fukami, T., and Shavelson, R. J. (2013) Context matters:Volunteer bias, small sample size, and the value of comparison groups in the assessment of research-based undergraduate introductory biology lab courses. J. Microbiol. Biol. Educ. 14, 176–182.
[7] Jones, M. T., Barlow, A. E., and Villarejo, M. (2010) Importance of undergraduate research for minority persistence and achievement in biology. J. Higher Educ. 81, 82–115.
[8] Brownell, S. E., Hekmat-Scafe, D. S., Singla, V., Chandler Seawell, P., Conklin Imam, J. F., et al. (2015) A high-enrollment course-based undergraduate research experience improves student conceptions of scientific thinking and ability to interpret data. CBE Life Sci. Educ. 1414, 2r21.
[9] Brownell, S. E., and Tanner, K. D. (2012) Barriers to faculty pedagogical change: Lack of training, time, incentives, and . . . tensions with professional identity?. CBE Life Sci. Educ. 11, 339–346.
[10] Kloser, M. J., Brownell, S. E., Chiariello, N. R., and Fukami, T. (2011) Integrating teaching and research in undergraduate biology laboratory education. PLoS Biol 9, e1001174.
[11] Lopatto, D., Alvarez, C., Barnard, D., Chandrasekaran, C., Chung, H. M., et al. (2008) Undergraduate research. Genomics Education Partnership. Science 322, 684–685.
[12] Harrison, M., Dunbar, D., Ratmansky, L., Boyd, K., and Lopatto, D. (2011) Classroom-based science research at the introductory level: Changes in career choices and attitude. CBE Life Sci. Educ. 10, 279–286.
Lopatto, D. (2007) Undergraduate research experiences support science career decisions and active learning. CBE Life Sci. Educ. 6, 297–306.

Freed-Pastor, W. A., and Prives, C. (2012) Mutant p53: One name, many proteins. Genes Dev. 26, 1268–1286.

Flaman, J. M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., et al. (1995) A simple p53 functional assay for screening cell lines, blood, and tumors. Proc Natl. Acad. Sci. USA 92, 3963–3967.

Amberg, D. C., Burke, D. J., Strathern, J. N. (2005) Methods in Yeast Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9, 671–675.

Skotheim, J. M., Di Talia, S., Siggiö, E. D., and Cross, F. R. (2008) Positive feedback of G1 cyclins ensures coherent cell cycle entry. Nature 454, 291–296.

Menendez, D., Inga, A., and Resnick, M. A. (2009) The expanding universe of p53 targets. Nat. Rev. Cancer 9, 724–737.

Bargonetti, J., and Manfredi, J. J. (2002) Multiple roles of the tumor suppressor p53. Curr. Opin. Oncol. 14, 86–91.

Ishioka, C., Frebourg, T., Yan, Y. X., Vidal, M., Friend, S. H., et al. (1993) Screening patients for heterozygous p53 mutations using a functional assay in yeast. Nat. Genet. 5, 124–129.

el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Definition of a consensus binding site for p53. Nat. Genet. 1, 45–49.

Silver, J. M., and Eaton, N. R. (1969) Functional blocks of the ad-1 and ad-2 mutants of Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 34, 301–305.

Ghim, C. M., Lee, S. K., Takayama, S., and Mitchell, R. J. (2010) The art of reporter proteins in science: past, present and future applications. BMB Rep. 43, 451–460.

Juels, D. H., Matthews, B. W., and Huber, R. E. (2012) LacZ beta-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. Protein Sci. 21, 1792–1807.

Chene, P. (2001) The role of tetramerization in p53 function. Oncogene 20, 2617, 2611.

Herraez, A. (2006) Biomolecules in the computer: Jmol to the rescue. Biochem. Mol. Biol. Educ. 34, 255–261.

Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev. 8, 1235–1246.

Noble, J. E., and Bailey, M. J. (2009) Quantitation of protein. Methods Enzymol. 463, 73–95.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., et al. (1999) A leucine-rich nuclear export signal in the p53 tetramerization domain: Regulation of subcellular localization and p53 activity by NES masking. Embo J 18, 1660–1672.

Diamandis, E. P., and Christopoulos, T. K. (1991) The biotin-(strept)avidin system: Principles and applications in biotechnology. Clin. Chem. 37, 625–636.

Bykov, V. J., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., et al. (2002) Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat. Med. 8, 282–288.

Bykov, V. J., and Wiman, K. G. (2014) Mutant p53 reactivation by small molecules makes its way to the clinic. FEBS Lett 588, 2622–2627.

Underwood, K. F., Mochin, M. T., Brusgard, J. L., Choe, M., Gnatt, A., et al. (2013) A quantitative assay to study protein:DNA interactions, discover transcriptional regulators of gene expression, and identify novel anti-tumor agents. J Vis Exp 78, e50512.