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Controlled Access of p53 to the Nucleus Regulates its Proteasomal Degradation by MDM2

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Abstract

The tumor suppressor p53 can be sent to the proteasome for degradation by placing its nucleocytoplasmic shuttling under ligand control. Endogenous p53 is ubiquitinated by MDM2 in the nucleus, and controlling the access of p53 to the nuclear compartment regulates its ubiquitination and proteasomal degradation. This was accomplished by the use of a “protein switch” that places nuclear translocation under the control of externally applied dexamethasone. Fluorescence microscopy revealed that sending protein switch p53 (PS-p53) to the nucleus produces a distinct punctate distribution in both the cytoplasm and nucleus. The nuclear role in accessing the proteasome was investigated by inhibiting classical nuclear export with leptomycin B. Trapping PS-p53 in the nucleus only allows this punctate staining in that compartment, suggesting that PS-p53 must translocate first to the nuclear compartment for cytoplasmic punctate staining to occur. The role of MDM2 binding was explored by inhibiting MDM2/p53 binding with nutlin-3. Inhibition of this interaction blocked both nuclear export and cytoplasmic and nuclear punctate staining, providing evidence that any change in localization after nuclear translocation is due to MDM2 binding. Further, blocking the proteolytic activity of the proteasome maintained the nuclear localization of the construct. Truncations of p53 were made to determine smaller constructs still capable of interacting with MDM2, and their subcellular localization and degradation potential was observed. PS-p53 and a smaller construct, construct containing the two MDM2 binding regions of p53 (Box I+V) were indeed degraded by the proteasome as measured by loss of enhanced green fluorescent protein that was also fused to the construct. The influence of these constructs on p53 gene transactivation function was assessed, and revealed that PS-p53 decreased gene transactivation, while PS-p53(BoxI+V) did not significantly change baseline gene transactivation.

Keywords

p53; MDM2; subcellular targeting; ubiquitin-proteasome pathway; protein switch

Introduction

Protein subcellular localization is tightly regulated in order for cells to control their function. Protein mislocalization has been implicated in a number of diseases,¹ and it has been proposed that correcting this mislocalization may be a potential treatment option.¹ It has also been found that inducing a change in a protein’s localization can be used to prevent that protein from functioning,² or can exploit that protein’s activity in a different compartment.³

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In an effort to control protein localization, our lab has developed technologies aimed at altering location and function.²⁻⁷ Recently, we have shown that a protein's function can be altered if it is sequestered in cytoskeletal aggregates,² or that apoptosis can be induced by sending p53 to the mitochondria.⁸ One of the technologies we have developed is the cytoplasm-to-nucleus protein switch (PS). This PS contains a nuclear export signal, a nuclear localization signal, and a ligand-inducible domain from the glucocorticoid receptor (GR). Ligand binding controls localization by a dexamethasone-specific ligand-binding domain (LBD) from GR, such that when expressed the protein is localized to the cytoplasm but is targeted to the nucleus upon induction by the ligand dexamethasone (dex).^{6, 7, 9}

p53 is a tumor suppressor that is maintained at low levels in normal cells, but rapidly accumulates in the nucleus in response to stress, such as DNA damage, hyperproliferation, chemotherapeutic agents, ultraviolet light, and hypoxia.¹⁰⁻¹⁴ The half-life of p53 is 6–20 minutes in healthy cells. However the concentration of p53 is increased 3- to 10-fold and the half-life is improved to hours in response to stress.^{10, 11} The accumulation is due to two factors: induced transcription of the *TP53* gene and reduced ubiquitination and proteasomal degradation.¹⁵ The accumulated p53 proteins are able to activate genes that promote growth arrest, apoptosis, and DNA repair through its transactivation function,¹⁶ and by inhibiting p53's degradation.¹⁷ MDM2 (HDM2 in humans) is the main protein responsible for the maintenance of p53, and does so with its RING finger domain E3 ligase activity that coordinates the transfer of ubiquitin from an E2 enzyme to lysine residues within p53.^{15, 18-23} Proteasomes recognize ubiquitinated proteins and degrade them.^{24, 25} However, overexpression of MDM2 results in downregulation of p53 and may lead to cell hyperproliferation, and this has been implicated in a variety of human cancers.^{26, 27} Small molecule inhibitors of the MDM2-p53 interaction have been developed to restore p53 activity, and may provide significant clinical benefit.²⁸⁻³⁰

Though the field of study of MDM2 and p53 is immense, the exact process of ubiquitination and subsequent degradation has not been fully elucidated. It is clear that conformation of p53, and the relative concentration of MDM2 to p53 greatly influence the ubiquitination fate of p53.^{20, 31} It is also known that MDM2 and p53 shuttle back and forth between the cytoplasm and nucleus via their respective NES and NLS, but that ubiquitination of p53 by MDM2 is mostly localized within the nucleus, and that access to both compartments is necessary for degradation.³² MDM2 can monoubiquitinate or polyubiquitinate p53; monoubiquitination exposes an NES in the C-terminal region of p53 and results in nuclear export, possibly with MDM2 in tow.^{31, 33} In the cytoplasm, MDM2 can efficiently polyubiquitinate p53, but it requires p53 to be in tetrameric form. Polyubiquitination can occur in the nucleus, and may favor proteasomal degradation within the nucleus.³¹

In this work, we hypothesized that p53 fused to our cytoplasm-to-nucleus protein switch could provide for a controlled interaction of p53 with MDM2, and thereby control the proteasomal degradation by ligand induction. We found that indeed controlling the access of p53 to the nucleus limited the apparent interaction of p53 to MDM2 to ligand induction, and we found this to be dependent upon nuclear translocation, nuclear export, and a functioning proteasome, with the ultimate outcome of proteasomal degradation of our construct. We also found that smaller domains of p53 could be fused to the protein switch with similar outcomes. To our knowledge, this is the first time that the interaction of p53 with MDM2 has been controlled by ligand induction, and represents an interesting method of targeting a protein to the ubiquitin-proteasome pathway.

Materials and Methods

Construction of EGFP-PS-p53

The DNA encoding p53 was amplified through PCR from pCMV-p53 wt (a generous gift from Dr. S. J. Baker, Addgene, Cambridge, MA) using the primers 5'-GCGCGCGGATCCGCCATGGAGGAGCCGCAGT-3' and 5'-GCGCGCGGATCCTCAGT-CTGAGTCAGGCCCTTCTGTC-3'. This was subcloned into the BamHI restriction enzyme site in the EGFP-HIV-MycA8-GRLBD (EGFP-PS) plasmid constructed previously.⁶ The EGFP-PS contains a nuclear export signal (NES) from HIV-rev protein,⁷ a nuclear localization signal (NLS) from MycA8 protein⁶ and a ligand binding domain from glucocorticoid receptor (GRLBD) with a point mutation (C656G) that makes it 10 times more sensitive to the agonist dexamethasone (dex).³⁴

Construction of Modified EGFP-PS-p53 Plasmids

EGFP-PS-p53ΔMBD: p53 was amplified without its MDM2 binding domain (MBD) through PCR using 5'-GCGCGCGCGCGGTACCGCTCCCAGAATGCCAGA-GGC-3' and 5'-GCGCGCGGATCCTCAGTCTGAGTCAGGCCCTTCTGTC-3' primers. This was subcloned into the KpnI and BamHI restriction enzyme sites in EGFP-PS.³⁴

EGFP-PS-p53ΔC

p53 was amplified without its C-terminal region by PCR using 5'-AATAATCTCGAGTTATGGAGGAGCCGCAGTCAG-3' and 5'-TAATAACTGCAGCCCAGCCTGGGCATCCTTG-3' primers, which introduce XhoI and PstI restriction enzyme sites. After PCR, the fragment and EGFP-PS plasmid were digested with these enzymes, and ligated together.

EGFP-PS-p53ΔTD

To remove the tetramerization domain (TD) the pre- and post- TD fragments were spliced together. The pre-TD fragment of p53 was amplified by PCR using 5'-AATAATCTCGAGTTATGGAGGAGCCGCAGTCAG-3' and 5'-TACGAACTGCAGTGGTTTCTTCTTTGGCTGG-3' primers, which introduce XhoI and PstI restriction sites. The post-TD domain was amplified with 5'-AATAATGGTACCTAAGGAGCCAGGGGGGAGCAG-3' and 5'-TAATAAGGGCCCCGTCTGAGTCAGGCCCTTCTG-3' primers, which introduce KpnI and ApaI restrictions sites. The fragments and plasmids were digested and ligated with their respective enzymes, and ligated together in a two-step process.

EGFP-PS-p53(Box I) and (Box I + V)

DNA oligonucleotides were synthesized by the DNA/Peptide Core Facility at the University of Utah. For Box I the sense and antisense fragments were 5'-TCGAGTTGAGCCCCCTCTGAGTCAGGAAACATTTTCAGACCTATGGAACTACTTCCTGAAAACAACCTGCA-3' and 5'-GGTTGTTTTTCAGGAAGTAGTTCCATAGGTCTGAAAATGTTTCCTGACTCAGAGGGGCTCAAC-3'. These oligos were annealed, and ligated between XhoI and PstI in the parent plasmid. For Box V, 5'-CTCGGAACAGCTTTGAGGTGCGTGTGTGCTGTCTGGGAGAGACGGGCC-3' and 5'-CGTCTCTCCCAGGACAGGCACAAACACGCACCTCAAAGCTGTTCCGAGGTAC-3' were annealed, and ligated between KpnI and ApaI restriction sites in the parent plasmid.

F270A Mutations

All template plasmids had the F270A mutation introduced using the QuickChange II Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with forward primer 5'-CTACTGGGACGGAACAGCGCTGAGGTG-CGTGTTTGTG-3' and reverse primer 5'-CACAAACACGCACCTCAGCGCTGTT-CCGTCCCAGTAG-3'.

The University of Utah Core DNA Sequencing Facility verified the sequences of all subcloned constructs.

Cell Lines and Transient Transfections

C127-derived murine mammary carcinoma cells (1471.1 cells)³⁵ (gift of G. Hager, NCI, NIH) were grown as monolayers in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin-glutamine (Invitrogen), and 0.1% gentamicin (Invitrogen). T47D human ductal breast epithelial tumor cells (ATCC, Manassas, VA), MCF-7 human breast adenocarcinoma cells (ATCC), and MDA-MB-231 human breast adenocarcinoma cells (a generous gift from Dr. David Bearss, University of Utah) were cultured in RPMI (Invitrogen) supplemented similar 1471.1 media. Additionally, T47D and MCF-7 cells were supplemented with 4 mg/L insulin (Sigma, St. Louis, MO). The cells were maintained in a 5% CO₂ incubator at 37°C. 7.5×10^4 cells for 1471.1, 1.0×10^5 cells for MDA-MB-231, and 3.0×10^5 cells for T47D and MCF-7 cells were seeded in 2-well live cell chambers (Nalge Nunc, Rochester, NY) for fluorescence microscopy. Transfections were carried out 24 hours after seeding using 1 pmol DNA and Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. Constructs were induced with 100 nM of the dex ligand, 24 hours after transfection for 1 hour, or as otherwise noted.⁶ MCF-7 cells were also used as an alternative breast cancer cell line to 1471.1 cells for degradation studies, and p53 reporter gene assay.

Fluorescence Microscopy

Approximately 24 hours after transfection, protein localization was viewed by fluorescence microscopy. For microscopy studies, cells were plated on a clear cover glass in six well plates (Greiner Bio-One Cellstar, Monroe, NC) or live cell chambers (Nalge Nunc, Rochester, NY). Prior to cell imaging, media was replaced with phenol red-free DMEM (Invitrogen) containing 10% charcoal-stripped fetal bovine serum (CS-FBS, Invitrogen). Images were acquired as previously,³ using an Olympus IX71F fluorescence microscope (Scientific Instrument Company, Aurora, CO) with high-quality narrow band GFP filter (ex: HQ480/20 nm, em: HQ510/20 nm) from Chroma Technology (Brattleboro, VT) with a 40× or 60× PlanApo oil immersion objectives (NA 1.00) on an F-View Monochrome CCD camera. For live-cell imaging, the microscope stage was maintained at 37°C with an air stream incubator (Nevtek ASI 400, Burnsville, VA). All experiments were repeated in triplicates (n=3) and 10 cells were analyzed for each time-point in each experiment. Green fluorescent images in this publication have been pseudocolored with ImageJ software, and brightness and contrast have been adjusted for visibility.

Treatment with LMB, Nutlin-3, and MG132

Cells were incubated with 10 nM leptomycin B (LMB, an inhibitor of nuclear export) 10 hours after transfection and 14 hours before ligand induction with dex followed by microscopy. Another population of transfected cells was incubated with 10 μM nutlin-3 one hour before ligand induction (Sigma, St. Louis, MO). MG132 (Sigma) was used at a concentration of 50 μM.

Flow Cytometry for GFP intensity

MCF-7 cells were treated with 10 mg/mL cycloheximide 24 h after transfection to inhibit protein synthesis.³⁶ Adding cycloheximide 24 hours post-transfection allows the cells to recover from transfection; 24 hours also allows for protein expression to occur. Cells were then treated with 100nM dex for 0, 2, 4, and 6 h. After treatment, cells were suspended in PBS and analyzed using the FACSCanto-II (BD-BioSciences, University of Utah Core Facility) and FACSDiva software.³⁷ Excitation was set at 488 nm and detected at 507 for EGFP intensity. The means from three separate experiments (n=3) were analyzed using one-way ANOVA with Bonferroni's post-hoc test within each time point.

Reporter Gene Assay

As previously⁸, 3.5 µg of each construct was co-transfected with 0.35 µg of pRL-SV40 plasmid encoding for *Renilla* luciferase (Promega, Madison, WI) as well as 3.5 µg of p53-Luc Cis-Reporter (Agilent Technologies, Santa Clara, CA) in MCF-7 cells. Cells were then treated with 100nM dex to be compared to no dex treated cells. *Firefly* and *Renilla* luminescence were detected via the Dual-Glo Luciferase Assay System (Promega) per manufacturer's instructions using the PlateLumino Luminometer (Strattec Biomedical Systems, Birkenfeld, Germany). The *Firefly* luciferase activity was normalized with *Renilla* luciferase values. The positive control (E-p53) was set at 100% and untransfected cells were set at 0%. The experiment was performed with an n=3 and analyzed using two-way ANOVA with Bonferroni's post-hoc test.

Results

p53 fused to the protein switch forms punctate distribution after ligand addition

Our previous studies have shown that the localization of exogenous proteins can be controlled by fusing a protein switch (PS) containing an NES, NLS, and ligand-binding domain (LBD).^{3, 6} In this study, we aimed to rationally design a protein switch that would target the proteasome; in the absence of ligand, it would localize in the cytoplasm, but after ligand addition (dexamethasone; dex), it would target the proteasome. We hypothesized that a PS-p53 fusion would target the proteasome when p53 was allowed to interact with MDM2, which is mainly localized within the nucleus.

The p53 cDNA was subcloned into the protein switch (PS) to create E-PS-p53. The enhanced green fluorescent protein (EGFP; E) tag facilitates localization tracking with fluorescence microscopy. After transient transfection in 1471.1 mouse mammary adenocarcinoma cells, the "empty" protein switch (E-PS) localizes in the cytoplasm (Figure 1 A, top left), but translocates into the nucleus after dex addition (Figure 1 A, top right). Wild-type p53 tagged to EGFP is unaffected by dex treatment, and appears to localize mostly in the nucleus (Figure 1 A, second row). The localization of transiently transfected p53 (E-PS-p53) was examined (Figure 1 A, third row) before and after dex treatment. Before drug, the protein had a mostly cytoplasmic localization, similar to E-PS. However within an hour of dex treatment, the protein took on a distinct punctate distribution in both the cytoplasm and nucleus. EGFP transfected cells (Figure 1A, bottom panel) were used as a negative control. There was no effect on EGFP localization before or after dex treatment.

E-PS-p53 was transiently transfected in human breast cancer cell lines MCF-7, T-47D, and MDA-MB-231 (Figure 1 B). E-PS-p53 appeared to follow the same response pattern to dex in the human breast cancer cells. However, ligand responsiveness was not observed in HeLa cells; the localization did not change with dex addition (data not shown). The punctate formation occurred within 30 minutes in 1471.1 cells after the addition of dex. A

representative set of pictures over 30 minutes at 150 nM dex dose for EGFP-PS-p53 is shown in Figure 2.

PS-p53 interacts with MDM2 in the nucleus before export

To investigate the mechanism behind the cytoplasmic and nuclear punctate localization of E-PS-p53, the effect of drug inhibitors of nuclear export (leptomycin B; LMB) and MDM2 interaction (nutlin-3) were examined. LMB is an inhibitor of CRM1 (chromosome region maintenance/exportin 1), which mediates classic nuclear export via nuclear export signals, including the nuclear export of wild-type p53.^{1, 38, 39} By inhibiting export, proteins localized within the nucleus are trapped there, but proteins not located—or shuttling to—the nucleus will be unaffected. The results of a 14 hour pre-incubation with 10 nM LMB, followed by 1 hour incubation with 150 nM dex, demonstrate that E-PS-p53 accumulates within the nucleus, showing the same punctate distribution only in the nucleus (Figure 3 A). The accumulation is due to the unavailability of the nuclear export machinery to shuttle the construct back to the cytoplasm. This confirms that the protein translocates to the nucleus upon dex treatment, before shuttling back to cytoplasm.

MDM2 is the main E3 ubiquitin ligase for p53, and it resides mainly in the nucleus. We already determined that E-PS-p53 first travels to the nucleus after dex addition (Figure 3A), so we speculated that the subsequent nuclear export and punctate distribution was due to MDM2 by facilitating ubiquitin conjugation to the p53 domain of our construct. Adding nutlin-3, a competitive inhibitor of the interaction of p53 with MDM2, elucidated the role of MDM2 in our system. Figure 2 B shows the results of nutlin-3 incubation in 1471.1 cells transfected with E-PS-p53, with or without dex addition. Without dex (left image), but with nutlin-3, the localization is no different from that seen without nutlin-3 incubation; the protein is localized mainly in the cytoplasm. However, dex induction in the presence of nutlin-3 (right image) demonstrates nuclear translocation but lacks any evidence of nuclear export, and the protein does not show punctate staining. The localization with nutlin-3 appears similar to E-PS with dex alone—the p53 domain does not appear to have an impact on localization. Taken together, these results suggest that E-PS-p53 responds to dex treatment by translocating to the nucleus where it interacts with MDM2, and subsequently shuttles back to the cytoplasm and takes on punctate distribution.

Inhibiting the proteasome alters PS-p53 localization

Wild-type p53 is sent to the ubiquitin-proteasome pathway by MDM2. To investigate whether the cytoplasmic and nuclear clusters were indeed constructs destined for the proteasome, we tested the effect of proteasomal inhibition on E-PS-p53 localization. 1471.1 cells were again transiently transfected with E-PS-p53 and incubated with the proteasomal inhibitor MG132 ± dex. MG132 did not appear to have an effect on the localization of the construct in the absence of dex (Figure 3 C, left image), but in the presence of dex, the construct demonstrated nuclear accumulation without visible clusters in the nucleus or cytoplasm (Figure 3 C, right image). Thus, a functioning proteasome was necessary for the nuclear export and punctate distribution of E-PS-p53 after dex treatment.

p53 truncations also interact with MDM2 in the nucleus

We sought to determine if smaller constructs comprised of p53 domains could produce the same localization change in response to ligand when fused to the protein switch. Figure 4 A shows representative images of select p53 truncations. A table of all tested constructs is shown in Figure 4 B. All constructs had varying degrees of cytoplasmic and nuclear localization upon dex addition, and some constructs did not result in proteasome pathway targeting. The constructs that did yield localization control similar to E-PS-p53 were E-PS-p53 Δ C, E-PS-p53 Δ MBD, and E-PS-Box I+Vmut. E-PS-TD only included the

tetramerization domain of p53, and showed equivocal targeting in microscopy studies. Interestingly, removal of the N-terminal MBD domain did not abrogate targeting. The F270A mutation (mut) has been previously described as leading to destabilization and hyperubiquitination and increased proteasomal degradation of p53 beyond the C-terminal region typically associated with polyubiquitination.⁴⁰ We included this mutation in several constructs to see if it imparted increased targeting to the proteasome pathway. The mutation did not universally increase proteasome pathway targeting, but it did for the E-PS-Box I+V construct.

The PS-p53 proteasomal degradation is under ligand control

To further examine the degradation of the PS-p53 upon ligand addition, MCF-7 cells were transfected with selected constructs followed by dex and cycloheximide treatments. Cycloheximide was used to ensure no additional protein synthesis³⁶ after the addition of dex to be able to quantify the GFP intensity and hence protein expression³⁷ before and after dex treatment. Expectedly, the negative controls (EGFP, E-p53, and E-PS-TD) did not show any change in GFP intensity over the 6 hour dex treatment (Figure 5). On the other hand, E-PS-p53 and E-PS-Box I+Vmut displayed decreased GFP intensity over time (Figure 5). E-PS-Box I+Vmut was degraded more significantly than E-PS-p53 at each time point. In addition, similar to the negative controls, the E-PS-p53 Δ C with dex treatment was not degraded despite its punctate formation.

PS-p53 constructs do not initiate transactivation via p53 promoter

In addition to its proteasomal targeting, we investigated the ability of p53 in the proteasomal switch to act as a transcription factor. Since the p53 cis-reporter system is widely utilized to evaluate the transcriptional activity of p53, MCF-7 cells were used to evaluate the activity of our proteasomal constructs before and after dex (Figure 6). The negative controls (EGFP and E-PS) reflected the endogenous transcriptional activity (all cells contain endogenous p53), which is significantly lower than the E-p53 treated cells (positive control). Both E-PS-p53 and E-PS-Box I+Vmut showed no additional activity. Similar to the negative controls, the E-PS-Box I+Vmut did not affect the endogenous transcriptional activity. However, the E-PS-p53, similar to E-PS-TD, showed lower transcriptional activity than the negative control.

Discussion

This work demonstrated that p53 could be fused to a protein switch capable of nucleo-cytoplasmic translocation under the control of external ligand. We took advantage of our nuclear protein switch (PS), which contains a ligand inducible nuclear import⁴¹ and an export signal to control the localization to the nucleus.⁴² The localization is controlled by a dex-specific LBD, which was cloned from the GR. When expressed, the protein is localized to the cytoplasm but is targeted to the nucleus upon ligand induction.^{6, 7, 9} This placed MDM2's access to p53, and subsequent ubiquitination and degradation, also under control of external ligand. To our knowledge, this is the first time that a protein's targeting to the ubiquitin-proteasome pathway has been controlled by regulating access to subcellular compartments. Knowledge of the ubiquitin-proteasome system is pivotal for understanding the degradation of proteins via the proteasome.⁴³ A protein that is destined for degradation goes through a post-translational modification called ubiquitylation, which is a process that covalently modifies proteins with ubiquitin via the enzymatic activity of an E1, an E2, and an E3 protein. Ubiquitin is first activated and transferred to the E1 active cysteine residue via a thioester linkage in a process requiring ATP. Ubiquitin is then transferred to the active cysteine residue of an ubiquitin-conjugating enzyme, E2, via a trans(thio)esterification reaction. Ubiquitin ligase, E3, interacts with both E2 and the

targeted protein. In general, E3 is important for mediating substrate specificity.⁴⁴ Subsequently, ubiquitin is ligated to the ε-amino group of a lysine of the target protein through a more stable isopeptide linkage. Since ubiquitin itself contains seven lysine residues, it allows the synthesis of polyubiquitin chains through isopeptide linkages. The process is then repeated giving rise to a polyubiquitin chain, which is recognized by the 19S regulatory caps of the proteasome. In the case of p53 degradation, MDM2 acts as the ubiquitin ligase (E3) while Ubc5 is the ubiquitin conjugating enzyme (E2).¹⁹ Degradation of p53 is dependent on the nuclear exclusion of p53 by MDM2.⁴⁵ However, some nuclear proteasomal degradation of p53 can be induced by MDM2.⁴⁶

The mechanism by which our proteasomal protein switch occurs is illustrated in Figure 7. We propose that when p53 translocates to the nucleus, it binds to MDM2 and is subsequently ubiquitinated, which results in cytoplasmic and nuclear punctate staining, possibly as ubiquitinated aggregates committed to proteasomal degradation. We confirmed that, indeed, PS-p53 did first travel to the nucleus before being exported to the cytoplasm by inhibiting nuclear export, which demonstrated nuclear accumulation of our construct (Figure 3A). When the interaction of MDM2 with p53 was inhibited by nutlin-3, whole-cell proteasome-pathway targeting was blocked (Figure 3B), verifying that MDM2 was involved in the ubiquitination process. Interestingly, in addition to preventing punctate distribution of our construct, blocking the proteolytic activity of the proteasome promoted nuclear localization (Figure 3C), possibly due to lack of ubiquitin availability. Interestingly, fusing the PS to p53 increased its availability to bind to MDM2 compared to E-p53, which shows minimum punctate formation (Figure 1) and no degradation compared to E-PS-p53 (Figure 5).

There are six lysine residues in p53 that get ubiquitinated by MDM2: K370, K372, K373, K381, K382, and K386.⁴⁷ The ubiquitination of p53 by MDM2 begins with the binding of MDM2 to the N-terminal Box-I region of p53. This single interaction is not enough for MDM2 to ubiquitinate p53; small molecule mimetics of this interaction do not prevent the E3 ligase activity of MDM2.^{18, 48–51} A second region within p53, called Box-V, was determined to be capable of providing a ubiquitination signal and docking site for MDM2.^{48, 52} This lead Wallace et al. proposed a model where binding of MDM2 to the N-terminal Box-I region of p53 allosterically promotes binding of the acidic domain of MDM2 to the Box-V region.^{52, 53} In other words, the allosteric activation of MDM2 by the N-terminal Box-I region allows a conformation in MDM2 to bind the Box-V region within p53's DBD to allow E2 transfer of ubiquitins to the C-terminus of p53.

Indeed, we found that isolating the regions of MDM2 binding (Box I+V) could target the proteasome pathway if a specific destabilizing F270A mutation was made (Figure 4A). This phenylalanine residue can block ubiquitination activity by MDM2, and its removal is associated with hyperubiquitination of p53.⁴⁰ The unmutated Box I+V construct lacks the tetramerization domain, which has previously been recognized as necessary for efficient polyubiquitination by MDM2.⁴⁰ The F270A mutation may overcome that inefficiency, which explains the degradation seen with Box I+Vmut (Figure 5). This construct might be advantageous over E-PS-p53 to future applications for several reasons. Smaller plasmids are easier to transfect, and will leave room for the fusion of other downstream domains. Also, it did not appear to block gene transactivation like full-length p53 fused to the protein switch (Figure 6), which might bind to and hence deactivate endogenous p53 because of its intact TD. This was confirmed with similar results when E-PS-TD was used (Figure 6). Other truncations of p53 also demonstrated visual proteasome pathway targeting, but these failed to demonstrate degradation. The reason for the difference in fate could be the interplay of monoubiquitination and/or polyubiquitination by MDM2 based on construct localization and dimerization potential.^{20, 54}

Like nature, the protein switch was designed to regulate cellular proteins by changing their location and hence, their function.^{55, 56} The protein switch has the advantage of being regulated by externally added ligand.^{1, 6, 7} The proteasomal protein switch can be engineered with a dimerization domain of a protein of interest, which will allow it to “capture” a cytoplasmic endogenous protein and send it for proteasomal degradation upon ligand induction. Since the ubiquitin ligase (E3) is what mediates substrate specificity,⁴⁴ we envision the use of the constructed proteasomal protein switch as a therapeutic to target oncogenic proteins. Depleting cytoplasmic oncogenic proteins such as Bcr-Abl, survivin, p27, and Raf-1^{3, 57–59} would be a specific treatment for cancer cells. This might prove useful in cancer with elevated levels of MDM2 such as breast cancer, melanoma, esophageal cancer, leukemia, sarcoma, non-small cell lung cancer, and non-Hodgkin’s lymphoma.²⁶ In addition, the EGFP-PS-p53 construct could also be used as a p53-MDM2 binding assay using fluorescence microscopy to screen for inhibitors that could disrupt the p53-MDM2 interaction. Nutlin-3, a MDM2 inhibitor, was used to confirm the feasibility of the assay.

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Figure 1.

A: Fluorescence microscopy of transiently transfected 1471.1 cells, with no drug treatment (left) and 150 nM dexamethasone (dex) at 1 hour (right). The protein switch, E-PS, localized in the cytoplasm without drug, but translocated to the nucleus after dex addition. Wild-type p53 tagged to EGFP (E-p53) did not respond to dex treatment, and had similar localization regardless of drug treatment. The protein switch fused to p53 (E-PS-p53) localized in the cytoplasm without dex, but demonstrated a distinct punctate pattern in the cytoplasm and nucleus. EGFP alone was localized to both cytoplasm and nucleus regardless of dex due to passive diffusion. B: E-PS-p53 transiently transfected in various cancer cell lines (MCF-7,

T47D, MDA-MB-231), with and without 1 hour of 150 nM dex, showing similar results to 1471.1 cells. Scale bars in A and B represent 20 μm .

Figure 2.

Representative time-dependent targeting for protein constructs. The figure shows representative time-dependent targeting for EGFP-PS-p53 with 150 nM dex. The movement of the protein could be observed from the cytoplasm to the proteasome over time upon ligand induction. White scale bars are all 20 μm.

Figure 3.

The effect of inhibition of nuclear export, MDM2, or proteasome was examined in 1471.1 cells transiently transfected with E-PS-p53. A: Cells incubated with LMB, an inhibitor of nuclear export, showed a cytoplasmic localization before dexamethasone (dex) treatment (left), but constructs demonstrated nuclear accumulation and punctate staining almost exclusively in the nucleus after 1 hour of 150 nM dex (right). B: Cells were incubated with an inhibitor of the interaction between MDM2 and p53. Without dex, nutlin-3 did not significantly change the baseline localization of E-PS-p53, but after 1 hour of 150 nM dex, the construct accumulated in the nucleus and did not show any punctate distribution. C: The role of the proteasome was evaluated by incubating cells with an inhibitor of the

proteasome, MG132. The inhibitor had no effect on the baseline localization of the construct, but after 1 hour of incubation with 150 nM dex, the construct took on a mostly nuclear localization, and showed a lack of cytoplasmic or nuclear punctate staining. Scale bars represent 20 μm .

B

Construct	Targeting
E-PS-p53	Y
E-PS-p53mut ¹	N
E-PS-p53 Δ C	Y
E-PS-p53 Δ TD ¹	N
E-PS-p53 Δ TDmut ¹	N
E-PS-TD	~
E-PS-p53 Δ MBD ¹	Y
E-PS-Box I ¹	N
E-PS-Box I + V	N
E-PS-Box I + Vmut	Y

¹Microscopy not shown

*F270A mutation

BI: Box I

BV: Box V

Figure 4.

A: Representative fluorescence microscopy images of 1471.1 cells transiently transfected with plasmids encoding truncated p53 constructs fused to the protein switch. The top row is no drug, whereas the bottom row is 1 hour of 150 nM dexamethasone (dex). E-PS-p53 Δ C, E-PS-p53 Δ MBD, and E-PS-Box I+Vmut constructs demonstrated proteasome pathway targeting; the TD construct was equivocal in regard to proteasome pathway targeting. B: Table of all created constructs, their schematic representation and whether they appeared to target the proteasome pathway. The F270A mutation represented as “mut”. Scale bar represents 20 μ m.

Figure 5.

The degradation of the expressed proteins was measured by quantifying the loss of fluorescence intensity of GFP. The GFP intensity was measured in transfected and dex treated MCF-7 cells and related to cells untreated with dex for each construct. E-PS-p53 and E-PS-Box I+Vmut were the only constructs showing reduction in relative GFP intensity with E-PS-Box I+Vmut being more significant at each time point. Mean values were analyzed using one-way ANOVA with Bonferroni's post-hoc test within each time point (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to EGFP). Error bars represent standard deviations from three independent experiments ($n=3$).

Figure 6.

Relative luminescence represents the transcriptional activity of p53 in MCF-7 cells. E-PS-Box I+Vmut showed no change in transactivation compared to negative controls (EGFP and E-PS), while E-PS-p53 exhibited reduction in transactivation similar to E-PS-TD. Mean values were analyzed using two-way ANOVA with Bonferroni's post-hoc test (** $p < 0.001$). Error bars represent standard deviations from three independent experiments ($n=3$).

Figure 7.

The proposed mechanism of proteasomal targeting of E-PS-p53. E-PS-p53 is expressed in the cytoplasm and is targeted to the nucleus upon ligand (dexamethasone) induction. MDM2 binds to p53 in the nucleus causing ubiquitination of p53. A small percentage of the ubiquitinated p53 is sent to nuclear proteasome for degradation. The majority of the ubiquitinated p53 is exported back to the cytoplasm and targeted to the cytoplasmic proteasome for degradation. LMB, nutlin-3, and MG132 can inhibit nuclear export, MDM2-p53 binding, and the proteasome, respectively.