# Mapping p53 proteoforms by native and denaturing top-down mass spectrometry

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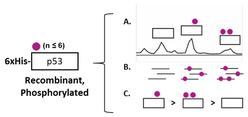
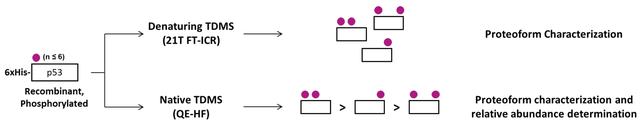
## OVERVIEW

**Objective:**



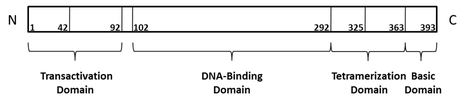

Our primary aim for these studies was to pursue training sets of representative rp53 proteoforms, in order to allow further optimization of chromatographic proteoform resolution and accurate mass determination (by high-resolution FTMS) **(A)**, complete PTM characterization by targeted MS2 fragmentation **(B)**, and relative proteoform abundance determination through a combined strategy of denaturing and native top-down mass spectrometry **(C)**.

**Method:**




## INTRODUCTION

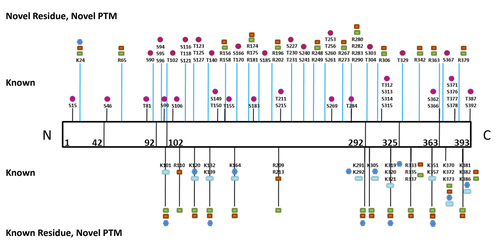
**The p53 protein: a master cellular regulator**




**67** known p53-dependent cellular processes
**458** clinically significant hp53 variations
**24** known functions of the p53 protein
**> 300** PTMs identified on p53
**133,426** PubMed entries

Theoretical Mass: **43,653.08 Da**

**Endogenous human p53 is extensively modified**

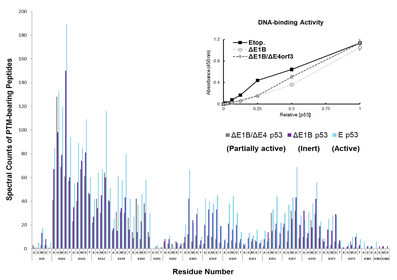



Summary of PTMs identified by tandem MS on endogenous human p53 isolated from primary fibroblasts (HFFs).

[Modified from DeHart C. J., Chahal, J. S., Flint, S. J., and Perlman, D. P., *MCP*, 2014.]

- Acetylation
- Di and/or Tri-methylation
- Methylation
- Phosphorylation
- Ubiquitinylation

**Endogenous p53 PTMs most likely comprise a combinatorial code**

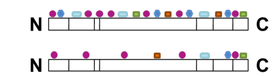



Summary and comparison of Lys modifications identified by tandem MS on three distinct populations of p53 isolated from HFFs: Active (**E p53**), Inert (**ΔE1B p53**), and Partially Active (**ΔE1B/ΔE4 p53**). Few differences in PTM location or identity could be determined, supporting the existence of a combinatorial PTM code governing p53 stability, localization, and transcriptional activity within the cell. **Inset:** The ability of the three p53 populations to bind to a synthetic consensus p53 sequence (one indicator for p53 transcriptional activity) was quantified via an ELISA-based assay.

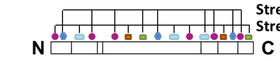
[Modified from DeHart C. J., Perlman, D. P., and Flint, S. J., *J. Virol.*, 2015.]

**Questions remaining after analysis of p53 by bottom-up mass spectrometry**


**How many combinatorial patterns of p53 PTM are realistically possible? (How many endogenous p53 proteoforms exist?)**

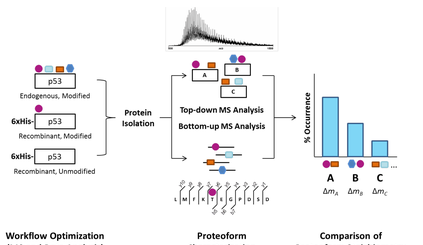



**How extensively modified is any single p53 proteoform?**




**How can the logic underlying p53 regulation via combinatorial PTM be deduced in the absence of proteoform identification?**

**Proposed analysis of endogenous p53 proteoforms by top-down mass spectrometry**

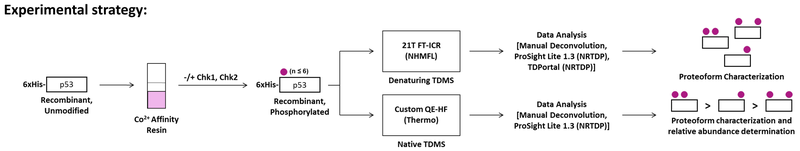



We hypothesized that the examination of endogenous and modified human p53 by top-down MS (TDMS) would enable both the generation of a comprehensive proteoform profile, comprising all proteoforms of p53 present within a specific cellular context, and the preliminary elucidation of the combinatorial regulatory logic underlying observed patterns of p53 PTM. To that end, we have developed a method by which training populations of recombinant and endogenous intact p53 protein can be isolated and analyzed for the presence of PTM by a combined strategy of high-resolution tandem MS and TDMS. Complete workflow optimization will enable the analysis and characterization of endogenous human p53 proteoforms from a broad range of cellular contexts, including multiple stress response pathways (e.g. DNA damage) and disease states (e.g. human cancers).

## METHODS

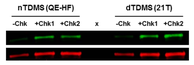
**Analysis of model p53 proteoforms by denaturing and native top-down mass spectrometry**

**Experimental strategy:**




Recombinant, full-length human p53 (rp53) was isolated from *E. coli* by cobalt ion affinity chromatography and phosphorylated *in vitro* by incubation with recombinant human Chk1 or Chk2 kinase (R&D Systems). Duplicate reactions (along with the kinase-free controls) were either subjected to native TDMS on a custom QE-HF mass spectrometer (Thermo) or resolved by RPLC and subjected to denaturing TDMS on a custom LTQ-Velos 21T FT-ICR mass spectrometer (National High Magnetic Field Laboratory, Florida State University). Additional preparations of unmodified rp53 were analyzed by denaturing TDMS on an LTQ Velos Orbitrap Elite (Thermo). Deconvolution of native and denaturing MS1 data was performed both manually and with MagTran, while analysis of the ensuing MS2 data was performed with ProSight Lite. Denaturing TDMS data were also searched against the human and *E. coli* proteomes using the Galaxy-based TDPortal available through the National Resource for Translational and Developmental Proteomics (NRTDP) at Northwestern University.

**Creation of model p53 proteoforms:**

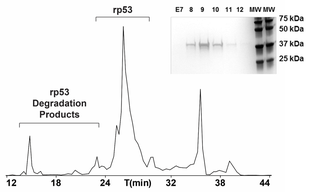



Visualization of rp53 phosphorylated in vitro by Chk1 or Chk2 kinase (ImageJ)
**ProQ:** Phosphorylated proteins
**SYPRO Ruby:** All proteins

## RESULTS

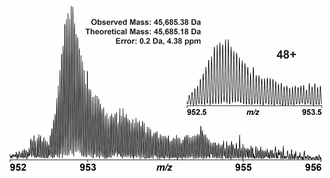
**Analysis of unmodified rp53 by denaturing top-down mass spectrometry**

**Example Chromatogram (21T FT-ICR):**



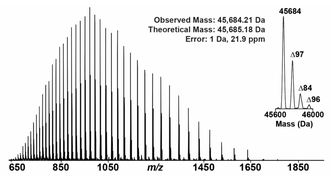

Total ion chromatogram (FTMS), showing low-MW rp53 degradation products and the majority peak containing full-length rp53. **Inset:** Eluted rp53-containing fractions were resolved by SDS-PAGE and visualized by $AgNO_3$.

**Isolated MS1 Spectrum (21T FT-ICR):**



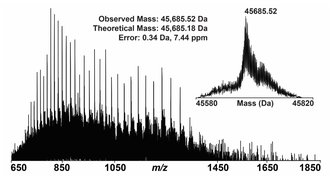

High-resolution isolated MS1 (FT) of the 48+ charge state of full-length rp53 (Avg. 2 scans), the first to be observed by LC-MS/MS at 21 tesla. **Inset:** Zoomed-in view of the two most abundant peaks, showing baseline isotopic resolution

**MS1 Spectrum (Orbitrap Elite):**



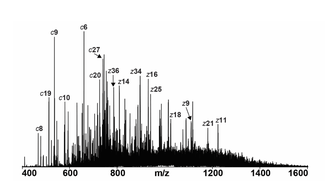

Medium-resolution MS1 (FT) of full-length rp53 (Avg. 10 scans). The observed mass is highly consistent with that obtained by *in silico* translation of the sequence-verified rp53 expression plasmid. **Inset:** Deconvoluted mass spectrum, demonstrating the complete resolution and mass determination of all rp53 proteoforms within the sample.

**Broadband MS1 Spectrum (21T FT-ICR):**



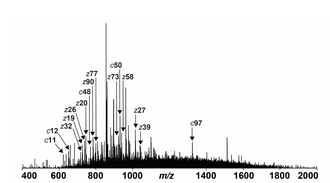

High-resolution broadband MS1 (FT) of full-length rp53 (Avg. 2 scans), the first to be observed by LC-MS/MS at 21 tesla. **Inset:** Deconvoluted mass spectrum, showing the isotopic resolution and exact mass determined for the most abundant rp53 proteoform within the sample.

**MS2 Spectrum (Orbitrap Elite):**




**Top:** Example MS2 spectrum of rp53 (Avg. 100 scans), obtained by ETD fragmentation of *m/z* 750 (± 50) for 10 ms, with annotation of select *c* and *z* fragment ion peaks. **Bottom:** Fragment map obtained by comparing the above fragment ions to the rp53 sequence in ProSight Lite. (P-score: 2 *e* -89)

**MS2 Spectrum (21T FT-ICR):**

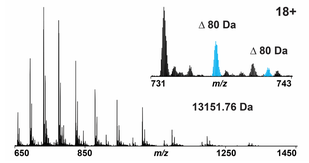



**Top:** Example MS2 spectrum of rp53 (Avg. 3 scans), obtained by ETD fragmentation of *m/z* 862.98 (± 7.5) for 7 ms, with annotation of select *c* and *z* fragment ion peaks. **Bottom:** Fragment map obtained by comparing the above fragment ions to the rp53 sequence in ProSight Lite. (P-score: 3 *e* -36)

## RESULTS II

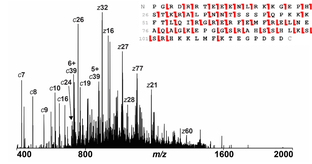
**Analysis of model p53 proteoforms by denaturing top-down mass spectrometry**

**Broadband MS1 Spectrum (21T FT-ICR):**




High-resolution single-scan MS1 (FT) of an rp53 degradation product phosphorylated by Chk1 and analyzed by LC-MS/MS at 21 tesla. **Inset:** Zoomed-in view of the 18+ charge state, with blue peaks indicating the two phospho-proteoforms of this 13.2 kDa species.

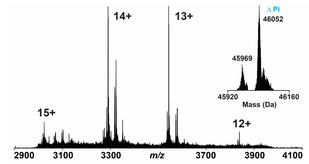
**MS2 Spectrum (21T FT-ICR):**




**Top:** Example single-scan MS2 spectrum of an rp53 degradation product phosphorylated by Chk1, obtained by ETD fragmentation of *m/z* 775.06 (± 7.5) for 10 ms, with annotation of select *c* and *z* fragment ion peaks. **Inset:** Fragment map obtained by comparing the above fragment ions to the rp53 sequence in ProSight Lite. (P-score: 3 *e* -88)

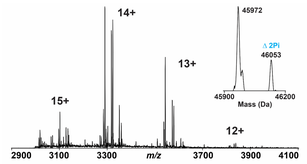
**Analysis of model p53 proteoforms by native top-down mass spectrometry**

**rp53 + Chk1: Native MS1**

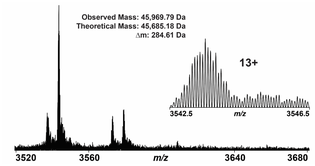



High-resolution MS1 (FT) of full-length rp53 phosphorylated by Chk1. **Inset:** Deconvoluted mass spectrum, showing the mass determined for the most abundant rp53 phospho-proteoform within the sample.

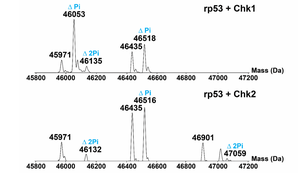
**rp53 + Chk2: Native MS1**




Low-resolution MS1 (FT) of full-length rp53 phosphorylated by Chk2. **Inset:** Deconvoluted mass spectrum, showing the mass determined for the most abundant rp53 phospho-proteoform within the sample.

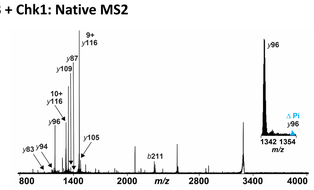



Zoomed-in view of the above FTMS1 spectrum, showing the 13+ charge state. **Inset:** Further zoomed-in view showing the baseline isotopic resolution of full-length phosphorylated rp53 obtained by native TDMS.

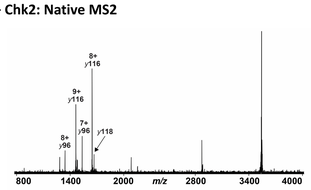



Comparison of deconvoluted native FTMS1 spectra of full-length rp53 phosphorylated by Chk1 or Chk2, showing the presence of multiple distinct rp53 phospho-proteoforms.

**rp53 + Chk1: Native MS2**




**rp53 + Chk2: Native MS2**



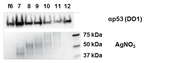

**Top:** Native MS2 spectrum of rp53 phosphorylated by Chk1, with annotation of select *b* and *y* fragment ion peaks. **Inset:** zoomed-in view of the y96 fragment ion, showing a second, phosphorylated *y*96 species. **Bottom:** Fragment map with hypothetical N-terminal phosphorylation site obtained by comparing the above fragment ions to the rp53 sequence in ProSight Lite. (P-score: 9 *e* -06)

**Top:** Native MS2 spectrum of rp53 phosphorylated by Chk2, with annotation of select *b* and *y* fragment ion peaks. **Bottom:** Fragment map with hypothetical N-terminal phosphorylation site obtained by comparing the above fragment ions to the rp53 sequence in ProSight Lite. (P-score: 1 *e* -04)

**Preliminary analysis of endogenous p53 populations by denaturing top-down mass spectrometry**

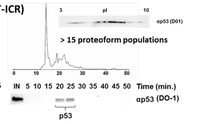
**Isolation of endogenous p53 via affinity chromatography:**


**Secondary MW-based separation via GELfrEE:**

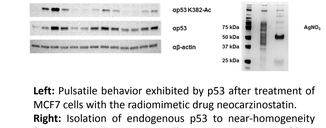



**Preliminary characterization of endogenous p53 proteoforms:**

**(12T FT-ICR)**




**Future Directions: endogenous human p53 proteoforms**




**Left:** Isolation of endogenous p53 to near-homogeneity from COS-1 cells.
**Right:** COS-1 p53 proteoforms were observed to elute indistinguishably over a 10-min. RT range. **Inset:** Isoelectric focusing of COS-1 p53 into >15 sub-populations.

**Left:** Pulsatile behavior exhibited by p53 after treatment of MCF7 cells with the radiomimetic drug neocarzinostatin.
**Right:** Isolation of endogenous p53 to near-homogeneity from MCF7 cells at 2 h.p.t. (first pulse shown on left) by immunoaffinity chromatography using a custom p53 resin.

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