Mutant p53 interactome identifies nardilysin as a p53R273H-specific binding partner that promotes invasion

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The invasiveness of tumour cells depends on changes in cell shape, polarity and migration. Mutant p53 induces enhanced tumour metastasis in mice, and human cells overexpressing p53R273H have aberrant polarity and increased invasiveness, demonstrating the 'gain of function' of mutant p53 in carcinogenesis. We hypothesize that p53R273H interacts with mutant p53-specific binding partners that control polarity, migration or invasion. Here we analyze the p53R273H interactome using stable isotope labelling by amino acids in cell culture and quantitative mass spectrometry, and identify at least 15 new potential mutant p53-specific binding partners. The interaction of p53R273H with one of them—nardilysin (NRD1)—promotes an invasive response to heparin binding–epidermal growth factor-like growth factor that is p53R273H-dependant but does not require Rab coupling protein or p63. Advanced proteomics has thus allowed the detection of a new mechanism of p53-driven invasion.

Keywords: HB–EGF; interactome; invasion; mutant p53; SILAC

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INTRODUCTION

p53 is a tumour suppressor protein whose gene (*TP53*) is frequently (>50%) altered in human cancers through missense mutations, resulting in high expression levels of full-length mutant protein [1]. Of the missense mutations reported in the IARC *TP53* database, the largest number of single base changes (>7.5% each) occur at amino acid positions 248 and 273 ((Database R15), [2]). Some of these mutations may be neomorphic or gain functions not normally exhibited by wild-type (wt)-p53 (reviewed in [3,4]), including invasion and metastasis as shown by studies of knock-in mice that express p53R270H (the equivalent of human p53R273H) [5–7]. These mice developed a different spectrum of tumours compared with p53^{-/-} mice including invasive and metastatic carcinomas [6]. Following ectopic expression of p53R273H and other p53 mutations in p53-null human cell lines, enhanced tumourigenicity in nude mice, colony formation in agar [8], increased proliferation [9] and augmented metastatic potentials [10,11] have been demonstrated.

Mechanisms underlying the oncogenic nature of mutant p53 include interaction or regulation of other transcription factors including p63, p73, NF-Y and VDR [12–15] or additional binding proteins such as TOPBP1, ANKRD11 and PIN1 [16–18]. Nearly all of the large number of interacting proteins reported in IARC p53 database [2] bind to wt-p53 and are therefore not good candidates for the neomorphic mutant hypothesis. To search for new mutant p53R273H-specific binding proteins, we undertook a comparative immunoprecipitation (IP) analysis using stable isotope labelling by amino acids in cell culture (SILAC) and mass spectrometry. SILAC has proved to be a powerful approach to distinguish adventitious from specific interactors, while retaining many weaker or transient binding partners [19–25]. Through this method we discovered several new mutant p53-specific binding proteins. Furthermore, we demonstrated the promotion of an invasive response to heparin binding–epidermal growth factor-like growth factor (HB–EGF) in mutant p53-expressing tumour cells that is dependent upon p53R273H and one of these mutant p53-specific interactors, Nardilysin (NRD1).

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Table 1 | Proteins that preferentially interact with p53R273H

Gene names	H/M (273H/Wt) ratio	Major domain/s	Related major pathways/processes
<i>Detected in only p53R273H IP</i>			
PAR3	13.0	PDZ	Asymmetrical cell division, cell polarization
MPDZ	12.4	PDZ, L27	Cell adhesion, epithelial polarity
NRD1	6.2	Peptidase_M16/M16_C	Cell proliferation, cell migration
HERC2	2.4	HECTc, CPH, MIB_HERC2, RCC1	Protein ubiquitination, intracellular protein transport
NT5C2	2.2	5_Nucleotid	Nucleotide metabolic process
NEURL4	2.0	Neuralized	Protein ubiquitination
PEO1	2.0	—	Nucleobase
PLOD1	1.8	2OG-FeII_Oxy	Metabolic process, lysine degradation
ATAD3B	1.7	AAA	Cell division
<i>Detected in both wt-p53 and p53R273H IP</i>			
GLT25D1	4.0	Glyco_trasf_25	Lipopolysaccharide biosynthetic process
PLOD3	3.7	P4Hc	Protein modification
RABGAP1	2.5	PH, TBC, SMC_prok_B	Cell cycle, regulation of Rab GTPase activity
PYCRL	2.1	—	Amino-acid biosynthetic process
TSPYL4	2.1	NAP	Nucleosome assembly
CCDC8	1.5	CCD	Regulation of protein amino-acid phosphorylation; protein complex assembly

RESULTS AND DISCUSSION

Mutant p53R273H and wt-p53 interactomes

The main aim of this study was to discover mutant p53-specific interacting proteins that lead to neomorphic functions in cancerous cells. For this we performed SILAC-based interactome analyses using high-resolution mass spectrometry. We carried out double- and triple-labelling SILAC experiments as described in Methods.

Using the high mass accuracy of the peptide precursor ion measurement, identification of SILAC pairs and the statistical significance calculated by the quantitative proteomics software, MaxQuant [26], it was possible to confidently differentiate the specific interacting partners of p53 from the normal population of nonspecific binding proteins (centered around '0' in the log₂ scale of the X axis in Fig 1B; supplementary Fig S1 online). From all experiments combined, we quantified 2052 proteins of which we identified 51 proteins in the wt-p53 IP and 64 in the p53R273H IP as potential interacting partners based on a normalized SILAC ratio ≥ 1.5 and significance factor of $P \leq 0.05$ present in at least two of the three experiments (supplementary Table S1 online). As expected, the majority of these interacting proteins were known to bind p53 or were contributors to network complexes (supplementary Fig S2 online).

New mutant p53R273H-specific binding partners

To distinguish mutant and wt-p53 preferential binding partners we compared and contrasted interacting proteins of p53R273H

and wt-p53 using triple-label SILAC IP (Fig 1A). In addition to detection in two out of the three p53R273H experiments, proteins that preferentially bound to the mutant were identified by H/M SILAC ratios (p53R273H/wt-p53) ≥ 1.5 and $P \leq 0.05$ in the triple-label experiment. To select new interacting partners, we chose proteins that have not been previously identified as p53 binding partners in the literature to the best of our knowledge. Using the above-mentioned criteria we identified 15 proteins that preferentially interacted with p53R273H, which are shown in Table 1, while those with the most significant SILAC ratios are depicted in Fig 1B. As we observed that some of these proteins were common to both mutant and wt-p53, we prioritized the mutant-specific interactors in Table 1 and focused on proteins that were only identified in the mutant IPs. Among them, Partitioning defective 3 homologue (PARD3 or PAR3), Multiple PDZ domain protein (MPDZ or MUPP1; supplementary Fig S3 online) and N-arginine dibasic convertase (NRD1; Fig 1C) were found to specifically bind to p53R273H, had the highest SILAC ratios and the most significant P -values. Consistent with the mass spectrometry results, western blot analysis showed that all these three proteins preferentially bound to p53R273H and not to wt-p53 (Fig 1D and supplementary Fig S3 online). Among the highly confident mutant-specific interactors, NRD1 was an interesting target to investigate further due its previously recognized role in invasion [27] but uncharacterized relationship to p53. To explore the specificity of this interaction, immunoprecipitated p53 complexes from a panel of mutant p53-expressing cell lines were probed with anti-NRD1. In all of the human tumour cell lines that expressed the R273H-mutant

p53 protein the presence of the NRD1–p53 complex was readily detected, but not from those cell lines expressing R280K- or R273C-mutant p53 protein (Fig 1E). The NRD1 interaction therefore defines a new class of mutant p53-specific binding protein that binds to only a subset of mutant p53 proteins and not to wt-p53.

p53R273H/NRD1-driven invasion towards HB-EGF

NRD1 is a metalloendopeptidase of the M16 family with widespread tissue expression, while higher levels are found in the testis, heart and skeletal muscle [28]. Although it is predominately localized in the cytoplasm, NRD1 has a nuclear localization signal through which it can shuttle between the nucleus and the cytoplasm [29]. Interestingly, NRD1 is also found in the pseudopodia of invading cells [30] and it has been implicated in cellular invasion towards HB-EGF [31]. As p53R273H has been shown to induce increased invasion in H1299 cells [11] and to further explore a role for NRD1 in p53R273H-mediated invasion, the three-dimensional movement of H1299 cells through a Matrigel matrix was examined (Fig 2). In the presence of HB-EGF and with control short interfering RNA (siRNA), twice the number of the cells expressing p53R273H invaded beyond 30 μm through a plug of Matrigel compared with p53-null control cells (Fig 2A,B). In contrast, following the knockdown of NRD1, the p53R273H-mediated invasion was lost while no effect was observed in control cells. These results indicate that NRD1 is important in p53R273H-dependent invasion in the presence of HB-EGF. This response, however, was specific to the use of HB-EGF as the chemoattractant, as the invasion of p53R273H-expressing cells towards EGF (as reported earlier [11]) was not inhibited by the siRNA knockdown of NRD1 (Fig 2C). The specificity of the NRD1–p53 interaction and its importance for the HB-EGF invasion response was further underlined by the finding that p53R280K, which cannot bind NRD1, was unable to promote invasion of H1299 cells towards the HB-EGF chemoattractant, while both p53R273H and p53R280K promoted invasion to EGF (Fig 2D,F). A further level of complexity was uncovered by examining the behaviour of the double-mutant p53 protein R273H/R280K. This protein was able to bind NRD1 (Fig 1E) but could not promote invasion of H1299 cells to HB-EGF (Figs 2E,F). The expression and functionality of this mutant p53 protein was confirmed by its ability to promote invasion towards EGF (Fig 2F). This indicates that the p53R273H–NRD1 association extends beyond a simple binding interaction, as the addition of the second R280K mutation abolishes its biological activity in this invasion assay.

To confirm our observations in tumour cells expressing endogenous p53R273H-mutant protein, we showed that U251 cells invaded in a mutant p53- and NRD1-dependent manner towards HB-EGF (Fig 3A,B). Interestingly, MDA MB231 cells that express the p53R280K mutation invaded in a p53-dependent and NRD1-independent manner towards fetal bovine serum (FBS; Figs 3C,D). This is consistent with the data from the transfected H1299 cells showing that invasion towards EGF depends on mutant p53 but not NRD1. Notably, MDA MB231 cells appear to be more sensitive to invasion towards FBS than U251 cells or p53R273H H1299 cells, which do not show invasion unless FBS-containing medium is supplemented with a chemoattractant (supplementary Fig S4 online). As predicted from the H1299 experiments, the MDA MB231 cells did not show enhanced

Fig 2 | Mutant p53- and NRD1-dependent invasion. (A) Western blot of H1299 control (V) and stable p53R273H-expressing cells treated with non-targeting control siRNA or NRD1 siRNA. Levels of NRD1 are shown with GCN5 as loading control. (B,C) For three-dimensional invasion studies, p53-null control (ctr, blue) and p53R273H (red)-expressing H1299 were assessed for invasion further than 30 μm through a Matrigel-fibronectin matrix towards HB-EGF (B) or EGF (C) in the presence or absence of NRD1 siRNA. The invasion in control cells transfected with control siRNA was set to 1. Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P = 2.8\text{E} - 4$, ** $P = 7.1\text{E} - 5$, *** $P = 4.4\text{E} - 3$ (Student's *T*-test). (D) Western blot of H1299 cells transiently transfected with the indicated mutant p53 cDNA or empty vector (V). (E and F) Assessment of relative (rel.) invasion similar as in panel B towards HB-EGF (E) or EGF (F) using H1299 cells transiently transfected with the indicated mutant p53 cDNA or empty vector (set to 1). Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P = 6.0\text{E} - 3$, ** $P = 7.7\text{E} - 4$, *** $P = 7.1\text{E} - 3$, **** $P = 8.7\text{E} - 4$ (Student's *T*-test). cDNA, complementary DNA; HB-EGF, heparin binding-epidermal growth factor-like growth factor; NRD1, nardilysin; siRNA, short interfering RNA.

invasion towards FBS supplemented with HB-EGF or invasion above background towards HB-EGF alone nor did loss of NRD1 or p53 impact this inability to invade (Fig 3E). As no interaction was detected between this endogenous R280K-mutant p53 and NRD1 (Fig 1E), these data further indicate that a complex between endogenous mutant p53 and NRD1 is required to drive invasion towards HB-EGF.

Fig 3 | Endogenous mutant p53 and NRD1 invasion. (A) Western blot of U251 cells treated with the indicated siRNA. Levels of NRD1 and p53 are shown with β -actin as loading control. (B) For three-dimensional invasion studies, U251 were treated with the indicated siRNA and assessed for invasion as in Fig 2B. Invasion of U251 cells treated with control (ctr) siRNA1 were set to 1. Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P=2.3E-5$, ** $P=2.7E-8$, *** $P=1.0E-8$, **** $P=3.6E-8$ (Student's *T*-test). (C) Western blot of MDA MB231 cells treated with the indicated siRNA. Levels of NRD1 and p53 are shown with β -actin as loading control. (D) For three-dimensional invasion studies, MDA MB231 were treated with the indicated siRNA and assessed for invasion further than 30 μ m through a Matrigel-fibronectin matrix towards FBS. The percentage of invading cells beyond 30 μ m through the matrix is represented. Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P=2.1E-4$ (Student's *T*-test). (E) MDA MB231 were treated with the indicated siRNA and assessed by confocal micrographs for invasion further than 30 μ m through a Matrigel-fibronectin matrix towards HB-EGF, HB-EGF + FBS, FBS alone or no attractant. The red dotted lines indicate 30 μ m. FBS, fetal bovine serum; HB-EGF, heparin binding-epidermal growth factor-like growth factor; NRD1, nardilysin; siRNA, short interfering RNA.

p53/NRD1-driven invasion is p63/RCP independent

Prior analysis of mutant p53-driven invasion towards EGF has shown that mutant p53 inhibits the p53 family member p63 and requires Rab coupling protein (RCP) to drive invasion. To support the concept that the p53R273H/NRD1-driven invasion towards HB-EGF acts through a distinct and new mechanism, the invasion properties of our H1299 cell models to HB-EGF after ablation of p63 and RCP were examined. Although loss of p63 increased invasion of p53-null H1299 cells to EGF [11], an effective p63 knockdown (Fig 4A) did not enhance invasion towards HB-EGF, confirming the mechanistic distinction between these two processes (Fig 4B). This distinction was further reinforced by establishing that RCP knockdown (which profoundly inhibits the EGF-driven invasion [11]) had no effect on the HB-EGF invasion response driven by the p53R273H mutant (Figs 4C,D). This analysis therefore demonstrates that one of the most common mutant proteins expressed in human cancer cells has a complex gain of function mediated by mutant-specific interactions. The p53R273H protein mediates invasion towards EGF through its ability to inactivate p63, while, as shown here, invasion to HB-EGF can be mediated through a new and highly specific biochemical interaction with NRD1.

Our observations that mutant p53 can cooperate with NRD1 to promote invasion are consistent with several recent studies [10–12,14,16–18,32] that demonstrated a neomorphic ability of mutant p53 to promote invasion. One mechanism underlying this gain of function of mutant p53 is an inhibition of the p53 family member p63, thereby promoting invasion responses to two important growth factors; EGF [11] and tumour growth factor- β

[10]. Here we show that invasion towards HB-EGF acts through a distinct pathway as it is not dependent on p63 (or RCP). HB-EGF, which has been shown to be a ligand for both the EGFR and the growth factor receptor HER4 [33], binds to NRD1 [31]. Our findings therefore support the possibility that some selective mutant p53 proteins can regulate multiple growth factor receptors to enhance invasion towards various chemoattractants, including EGF, tumour growth factor β and HB-EGF through distinct mechanisms. The direct physical interaction of p53R273H with NRD1 may affect HB-EGF interaction with its receptors to promote an invasive response, although our mutation analysis suggests that interaction alone is necessary but not sufficient for the biological response. These results encourage further detailed study of growth factor receptors, HB-EGF ligand and p53 interactions, including NRD1.

CONCLUSION

In conclusion, through the use of SILAC and comparative IPs we identified several new p53 binding proteins, including ones that specifically interacted with mutant p53 and not wt-p53. We confirmed a p53R273H-specific interaction with NRD1 and demonstrated that this cooperation was important in the promotion of cellular invasion driven by p53R273H towards HB-EGF. In addition to NRD1, we report at least 15 new potential mutant p53-specific binding partners that will be valuable in elucidating further the neomorphic functions of mutant p53 and strongly support the concept that distinct mutations in p53 may have selective new functions. Hence, inhibiting the expression or activity of mutant p53 proteins and modulating mutant

Fig 4 | p53- and Rab coupling protein (RCP)-dependent and independent invasion. (A) Relative amount of p63 mRNA as determined by quantitative reverse transcription-PCR analysis in H1299 p53-null cells treated with non-targeting control siRNA (set to 1) or p63 siRNA. Values are means $\pm$ s.e.m. of three independent experiments. * $P = 1.2E - 6$ (Student's *T*-test). (B) For three-dimensional invasion studies, H1299 p53-null cells were treated with non-targeting control (ctr) siRNA (set to 1) or p63 siRNA as indicated and assessed for invasion similar to Fig 2B towards HB-EGF as a chemoattractant. Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P = 5.0E - 4$ (Student's *T*-test). (C) Western blot of H1299 control (V) and stable p53R273H-expressing cells treated with non-targeting control siRNA or RCP siRNA. Levels of RCP are shown with β -actin as loading control. (D) For three-dimensional invasion studies, p53-null control (blue) and p53R273H (red)-expressing H1299 were assessed for invasion similar as Fig 2B towards HB-EGF in the presence or absence of RCP siRNA. Values are means $\pm$ s.e.m. of nine replicates from three independent experiments. * $P = 1.3E - 3$ (Student's *T*-test). HB-EGF, heparin binding-epidermal growth factor-like growth factor; siRNA, short interfering RNA.

p53-specific binding partners becomes an exciting and defined therapeutic approach to reduce tumour invasion and metastasis.

METHODS

Cell culture. The cells were cultured in either normal or SILAC media as described in supplementary information online. In SILAC experiments, mutant- and wt-p53-expressing cells were differentially labelled using SILAC to incorporate isotopic forms of lysine and arginine present in the DMEM media. For double-labelling experiments, the vector-only control cells were grown in media containing normal (or 'light' (L)) isotopes of lysine and arginine, while heavy (H) media was used for cells expressing p53. For the triple-label experiments, the vector was grown in light medium, the p53R273H-expressing cells in heavy and the wt-p53 cells in media containing an intermediate isotope (or 'medium' (M)). To ensure that the cells were treated in a similar

manner during the triple labelling, all were transiently transfected after stable isotope incorporation.

Immunoaffinity purification of protein complexes. Cells were lysed in modified RIPA buffer and the protein complexes were immunoprecipitated using either anti-FLAG or anti-p53 antibody as detailed in supplementary information online. Protein complexes were eluted either using $3 \times$ FLAG peptides or SDS buffer. Either the affinity-purified protein complexes were subjected to liquid chromatography-mass spectrometry analysis as described below or they were used for western blot analysis.

Mass spectrometry and data analysis. Eluted protein complexes were separated by 1D SDS-PAGE, and digested with trypsin using published procedures [34]. Samples were analysed on an Orbitrap or Orbitrap XL (Thermo Fisher) coupled to a Proxeon Easy-nLC under the conditions described in supplementary information online. Mass spectrometry data were analysed by MaxQuant version 1.0.13.13 [26] using default parameter settings. Maximum false discovery rates were set to 0.01 for both protein and peptide.

Invasion assays. Matrigel assays were performed as described previously [11,35] and in the supplementary information online. Briefly, cells were seeded on the base of transwell chambers and invasion towards a gradient of 10% FBS combined with 10 ng/ml HB-EGF or 25 ng/ml EGF (unless stated otherwise), and was measured by confocal microscopy in serial sections. Quantification of Matrigel images was performed in ImageJ. siRNA transfections for NRD1, p53, p63 or RCP were carried out using standard and according to manufacturers' procedures as described in supplementary information online. The silencing effect was assayed by western blots or reverse transcription-PCR or both.

Mass spectrometry data sets were deposited in PeptideAtlas under PASS00051 data set identifier (https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/PASS_View?identifier=PASS00051&browseArea=&path=/p53%20mutant-wt%20data%20sets).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: C.R.C., D.P.L. and J.G. conceived, designed and interpreted SILAC and mass spectrometry experiments and wrote the paper. C.R.C. with S.P.N.'s assistance carried out the I.P. experiments and western blots. P.A.J.M. and K.H.V. designed, performed and interpreted the cellular invasion assays and assisted in manuscript preparation. K.A.H., W.P.B. and J.G. performed and supervised the mass spectrometry analysis. H.K.O. and P.A.J.M. generated stable mutant p53 cell lines, while C.F.C. provided reagents and conceptual opinions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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