NEK10 tyrosine phosphorylates p53 and controls its transcriptional activity

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Abstract
In response to genotoxic stress, multiple kinase signaling cascades are activated, many of them directed towards the tumor suppressor p53, which coordinates the DNA damage response (DDR). Defects in DDR pathways lead to an accumulation of mutations that can promote tumorigenesis. Emerging evidence implicates multiple members of the NimA-related kinase (NEK) family (NEK1, NEK10, and NEK11) in the DDR. Here, we describe a function for NEK10 in the regulation of p53 transcriptional activity through tyrosine phosphorylation. NEK10 loss increases cellular proliferation by modulating the p53-dependent transcriptional output. NEK10 directly phosphorylates p53 on Y327, revealing NEK10’s unexpected substrate specificity. A p53 mutant at this site (Y327F) acts as a hypomorph, causing an attenuated p53-mediated transcriptional response. Consistently, NEK10-deficient cells display heightened sensitivity to DNA-damaging agents. Further, a combinatorial score of NEK10 and TP53-target gene expression is an independent predictor of a favorable outcome in breast cancers.

Introduction
Eukaryotic cells respond to various forms of DNA damage through distinct DNA damage response (DDR) pathways.

These molecular circuits first function to recognize damaged DNA and suppress cell cycle progression, to provide time for repair of genetic material or, if the damage is deemed irreparable, carry out an apoptotic programme. Deregulation of DDR networks leads to genetic abnormalities, chromosomal instability, and accumulation of mutations that can initiate neoplastic transformation and, ultimately, tumorigenesis. DDR networks are subject to complex regulation integrating multiple input signals to titrate the cellular response to DNA damage. One of the master regulators of the cellular response to DNA damage is the tumor suppressor p53, a transcription factor that coordinates an extensive gene expression program, both basally and in response to various forms of cellular stress. p53 transcriptional targets govern a wide range of cellular processes, including cell-cycle control, apoptosis, senescence, DNA repair, metabolism, immune response, and migration [1–4]. Loss of p53 can lead to genomic instability and acquisition of oncogenic mutations that effect proliferation, transformation, therapeutic resistance, and metastasis [2, 5]. TP53 germline mutations are the cause of the hereditary disorders: Li-Fraumeni syndrome and Li-Fraumeni-like syndromes, rare diseases that lead to predisposition to early onset cancers [6]. Somatic mutations of TP53 represent some of the most frequent alterations in human cancers with an incidence >50% (http://p53.iarc.fr/). The p53 protein is
modulated through multiple posttranslational modifications, including phosphorylation, ubiquitination, methylation, acetylation, and SUMOylation, in response to even low levels of DNA damage or stress, that act to direct and titer the cellular p53 response and elicit context-dependent phenotypic responses [3, 7–10].

Alongside p53, multiple other inputs, and signaling pathways have been implicated in the control of the cellular response to DNA damage and cell cycle checkpoint control, including members of the NEK kinase family. In response to ionizing radiation (IR), NEK1 and NEK11 activity supports checkpoint integrity and DNA repair, whereas inactivation of NEK2 or NEK6 is required for proper checkpoint engagement [11–18]. Both NEK8 and NEK9 were shown to participate in DNA repair and response to replicative stress [19–21], whereas NEK10 has been found to control G2/M checkpoint integrity in response to ultraviolet irradiation [22].

Cancer genomes analyses have correlated NEK10 status with cancer incidence and outcome. NEK10 alterations and mutations are found in ~2.6% of human cancers in the TCGA Pan-Cancer dataset [23–26]. A comprehensive genome-wide association study identified a strong breast cancer susceptibility locus within a sub-region of human chromosome 3p24 containing NEK10 [27], whereas lowered NEK10 expression associates with poor breast cancer prognosis and higher tumor grade [22]. Nevertheless, little is known about the function and molecular mechanism of action of NEK10 in tumorigenesis.

Here, we demonstrate a function for NEK10 in the regulation of p53 transcriptional activity via phosphorylation of Y327 within the p53 oligomerization domain. NEK10-deficient cell lines revealed a p53-dependent function of NEK10 in control of cellular growth, DNA replication, and sensitivity to genotoxic stress. Genomic analyses of breast cancer patient data identify NEK10 expression in combination with a p53-target gene signature as a candidate prognostic indicator in WT TP53 tumors.

Results

Loss of NEK10 leads to increased proliferation and DNA replication

To investigate the biological function of NEK10, we generated A549 lung adenocarcinoma (WT TP53) cell lines with a loss of NEK10 function by CRISPR-Cas9 mediated deletion of exon 24 of the NEK10 gene (A549 NEK10ΔΔ cells). The targeted exon contains the “DFG” motif, which is required for NEK10 protein kinase activity (Fig. S1). Targeting of this exon leads to a total loss of NEK10 mRNA expression (Fig. S2). Phenotypic characterization of NEK10ΔΔ cells, revealed an increase in proliferation and colony-forming ability, and a decrease in doubling time compared with both parental A549 cells and to NEK10Δ/+ single-cell clones (Fig. 1a, b, S3a), suggesting a growth-suppressive function for NEK10.

Despite the differences in cellular proliferation, cell cycle distributions based on propidium iodide (PI) staining of NEK10Δ/+ and NEK10ΔΔ cells were indistinguishable, pointing to intact basal checkpoint fidelity in the absence of NEK10 (Fig. 1c). Considering that PI staining of asynchronous cells only provides a “snapshot” of the DNA content of a population of cells, we pulse-labeled cells with 5-ethynyl-2′-deoxyuridine (EdU), a nucleoside analog of thymidine that is incorporated into the DNA during active DNA synthesis. Proportion of cells with active DNA synthesis (EdU+ cells), was increased in NEK10ΔΔ cells (Fig. 1d). Based on staining of cells for phosphorylated Histone H3 (Ser10), a mitotic marker, a higher proportion of NEK10ΔΔ cells were undergoing mitosis, further supporting a function for NEK10 in the control of cellular proliferation (Fig. 1e). The observed differences in cellular proliferation were not due to differences in cell death under normal cycling conditions, as loss of NEK10 did not affect the proportion of Sub-G1 cells (Fig. 1f).

NEK10 influences transcription of p53-target genes in a kinase-dependent manner

To explore the mechanism of NEK10-mediated control of cellular proliferation and DNA synthesis, we determined the cellular levels of p53 and the p53-responsive genes p21 and MDM2, which have established functions in control of these processes [28–31]. When compared with parental A549 and NEK10Δ/+ cells, p21 and MDM2 protein levels were lower in NEK10ΔΔ cells (Fig. 2a, S4a), without alterations in their turnover, as their half-lives were indistinguishable between wildtype and NEK10ΔΔ cells (Fig. S5). We next explored the mRNA levels of multiple p53-target genes (p21, p53, MDM2, GADD45α, PUMA, and BAX) by Quantitative reverse transcription PCR (RT-qPCR) and found decreased mRNA expression of p53-responsive genes. The greatest decreases were found in p21 and MDM2 gene expression in NEK10ΔΔ cells, followed by GADD45α, PUMA, BAX, and p53 (Fig. 2b), results corroborated in MCF-7 cells (WT TP53) deficient for NEK10 (Fig. S6). This effect was largely dependent on p53, as shRNA mediated knockdown of p53 led to decreases in the mRNA expression of p53-responsive genes in both NEK10Δ/+ and NEK10ΔΔ cells (Fig. 2c). Consistent with this observation, re-expression of NEK10 in NEK10ΔΔ cells led to an increase in the expression of both p21 and MDM2, while leaving p53 levels unaffected, indicating a relationship between NEK10 and p53 transcriptional activity (Fig. 2d, S4b).

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To explore the mechanism of p53 regulation by NEK10, NEK10ΔΔ cells were stably reconstituted with WT NEK10, a kinase activity-dead mutant NEK10 D655N, or a serine-restricted/tyrosine kinase activity-dead mutant NEK10 I693P (Fig. S7 and described in [32]). While overexpression of WT NEK10 induced a strong decrease in cellular proliferation (Fig. 3a, S3b), neither NEK10 D655N nor NEK10 I693P led to considerable growth suppression, supporting the notion that NEK10 kinase activity, and specifically its tyrosine kinase activity, was responsible for control of proliferation. NEK10-dependent growth suppression was coupled to an increase in G1/S arrest, without a change in the proportion of Sub-G1 cells (Fig. 3b). Consistent with this, only the cells expressing WT NEK10 displayed elevated expression of p21 and MDM2 (Fig. 3c, d, S4c). This NEK10 kinase activity-dependent effect on the expression of p53-responsive genes was indeed closely linked to p53 status, as WT NEK10 re-expression led to an increase in p53 levels (Fig. 3c).

**Fig. 1 NEK10 loss leads to an increase in cellular proliferation and DNA replication.** a Proliferation curve of A549 NEK10+/+ and A549 NEK10ΔΔ cells measured using SRB assay over a 72 h time period. Relative growth was calculated relative to the absorbance at t = 0 for each cell line (p < 0.002, two tailed t-test, n = 3 biological replicates, bars represent SEM). b Colony-forming assay of NEK10+/+ and NEK10ΔΔ A549 cells. 100 cells of each genotype were seeded and their plating efficiency determined (p < 0.05, two tailed t-test, n = 3 biological replicates, bars represent SEM). c Cell cycle distribution was determined using propidium iodide staining for total DNA content. G1, S, and G2 fractions were distinguished using FlowJo (p = ns, n = 3 biological replicates, two tailed t-test, bars represent SEM). d NEK10+/+ and NEK10ΔΔ A549 cells were pulse labeled with EdU for 2 h. Flow cytometry was performed to quantify the proportion of cells with active DNA replication (p < 0.05, n = 3 biological replicates, two tailed t-test, bars represent SEM). e Asynchronous cells were stained with a phospho-H3 (Ser10) antibody to determine the proportion of mitotic cells (p < 0.01, two tailed t-test, n = 3 biological replicates, bars represent SEM). f The proportion of apoptotic cells, as determined by propidium iodide staining, and quantification of the Sub-G1 cell proportion (p = ns, two tailed t-test, n = 6 biological replicates, bars represent SEM).
Fig. 2 NEK10 status modulates the expression of p53-responsive genes.

a Immunoblot of A549 NEK10+/+ and NEK10Δ/Δ cells for the expression of the indicated proteins. b RT-qPCR of p53-responsive genes in NEK10+/+ and NEK10Δ/Δ A549 cells (*p < 0.05, **p < 0.01, ***p < 0.001, two tailed t-test, n = 7 biological replicates, bars represent SEM). c RT-qPCR quantification of p53-responsive genes in the indicated A549 cell lines. Fold change is calculated relative to NEK10+/+ shGFP cells (*p < 0.05, **p < 0.01, ***p < 0.001, two tailed t-test, n = 3 biological replicates, bars represent SEM). d Immunoblot of NEK10Δ/Δ A549 cells reconstituted with indicated lentiviral constructs.

in p21 expression in NEK10Δ/Δ shGFP cells, whereas in NEK10Δ/Δ shp53 cells, p53 knockdown suppressed the extent of the WT NEK10-dependent p21 expression increase (Fig. 3e).

NEK10 phosphorylates p53 on tyrosine 327

Given the observed contribution of NEK10 kinase activity to the expression of p53-target genes and growth suppression, we explored if NEK10 can directly phosphorylate p53. In in vitro kinase assays, FLAG-NEK10 purified from HEK293T cells readily phosphorylated GST-p53 (Fig. 4a). Further, overexpression of NEK10 in HEK293T cells led to an increase in tyrosine phosphorylated p53, indicating that potential site of phosphorylation is a tyrosine residue (Fig. 4b). Tyrosine phosphorylated p53 (pY-p53) was detectable in both NEK10+/+ and NEK10Δ/Δ cells (Fig. 4c). However, in response to IR radiation, pY-p53 increased only in NEK10+/+ cells, indicating a function for NEK10 in tyrosine phosphorylation of p53 induced by genotoxic stress (Fig. 4c).

We next queried the Phosphosite™ database for reported sites of p53 tyrosine phosphorylation found in phospho-mass spectroscopic studies and identified three sites as possible candidate NEK10 target sites: Y126, Y220, and Y327. As both Y126 and Y220 had been previously characterized as sites of SRC-mediated phosphorylation [33–35], we focused on Y327 as a candidate site for NEK10 phosphorylation (Fig. 4d). Of note, Y327 fits within a recently mapped phosphorylation site motif for NEK10, with an aromatic phenylalanine at the P + 1 position [36]. NEK10 phosphorylated a peptide encoding p53 amino acids 320–335, but failed to phosphorylate the same peptide with a Y327F substitution (Fig. S8). Despite the peptide containing two candidate phospho-acceptor sites (Y327 and T329), its phosphorylation by NEK10 appeared to be wholly dependent on Y327 as a phospho-acceptor residue, as NEK10 readily phosphorylated the T329A mutant peptide. NEK10 failed to phosphorylate full length Y327F GST-p53, reinforcing Y327 as a site for NEK10 phosphorylation (Fig. 4e, f). Consistent with an in vivo function of NEK10 in p53 Y327 phosphorylation, WT p53 was tyrosine phosphorylated in NEK10+/+ but not NEK10Δ/Δ cells, whereas the p53 Y327F mutant was not tyrosine phosphorylated in either of the cell lines (Fig. 4g).

NEK10 phosphorylation of p53 tyrosine 327 supports p53 transcriptional activity

We next investigated the function of Y327 in p53 transcriptional activity, by transfecting HCT116 p53−/− cells with WT p53, p53 Y327F, and p53 L344P. Judging by transactivation of a panel of p53-target genes and the protein levels of p21 and MDM2, p53 Y327F was not a full loss-of-function p53 mutant but rather acted as a hypomorphic to WT p53 (Fig. 5a, b). Y327F substitution impaired p53 transcriptional activity, but to a lesser extent than the one caused by the L344P mutation, which causes complete loss of p53 transcriptional activity (Fig. 5a, b) [37]. Even though Y327 localizes to the oligomerization domain of
p53, and previous data indicating that nitration of this amino acid influenced oligomerization and transcriptional activity, Y327F mutation failed to impact p53 oligomerization in our system (Fig. 5c) [38, 39].

To determine if the effect of Y327F mutation on p53 transcriptional activity was dependent on NEK10, H1299 cells (p53-null) co-expressing p53 WT, p53 Y327F, or p53 L344P, respectively, in combination with a control FLAG vector or FLAG-NEK10, respectively, were assessed for induction of a panel of p53-responsive genes (Fig. 5d, e, S9). Although there was no difference in the expression of p53-responsive genes following p53 Y327F or WT p53 transfection, NEK10 co-expression led to a significant increase in p53-responsive genes only in cells expressing WT p53, but not p53 Y327F (Fig. 5d, e, S9).

Thus, NEK10 induces p53 transcriptional activity in a p53 Y327-dependent manner.

We further assessed p53 Y327 and NEK10 kinase activity relationship by co-expression of WT, kinase dead (A546F), or serine-restricted/tyrosine kinase activity-dead (I693P) NEK10 with WT p53 or p53 Y327F, respectively, in H1299 cells (Fig. 5f). As previously noted, p21 protein levels were only increased by co-expression of WT p53 and WT NEK10, whereas kinase-deficient NEK10 mutants (A546F, I693P) failed to affect p21 levels in combination with WT p53 (Fig. 5f). The NEK10 kinase activity effect on p53 transcriptional activity was Y327-dependent, as Y327F p53 mutants failed to induce p21 in the presence of either active or kinase-deficient NEK10 mutants (Fig. 5f).
Induction of p53-target genes in response to genotoxic stress requires NEK10

In light of NEK10’s impact on p53 transcriptional activity and previous work implicating NEK10 in the DDR [22], we next investigated the effect of NEK10 loss in the context of DNA damage. In response to cisplatin treatment, NEK10 loss impaired induction of certain p53-responsive genes, such as p21, GADD45α, and PUMA (Fig. 6a, b, S10). This effect was independent of p53 S15 phosphorylation, fluctuations in p53 protein stability or changes in p53 gene expression, as these remained indistinguishable between NEK10+/+ and NEK10Δ/Δ cells (Fig. 6a, b, Fig. S10). This defect in p53 transcriptional output was also found in response to IR, as irradiation led to a rapid and sustained increase in both p21 and p53 protein levels in the NEK10+/+...
NEK10 phosphorylates p53 on Y327 in vitro and in cells. a Radioactive in vitro kinase assay performed using purified FLAG-NEK10 WT and kinase dead FLAG-NEK10 (A546F) incubated with GST alone or GST-p53. b HEK293T cells transfected with the indicated constructs were lysed under denaturing conditions and tyrosine phosphorylated proteins were immunoprecipitated. The amount of pY-p53 was determined by anti-p53 immunoblotting. c NEK10 WT and kinase dead FLAG-NEK10 (A546F) incubated with p53 was determined by anti-p53 immunoblotting. Phosphorylated proteins were immunoprecipitated. The amount of pY-p53 was determined by anti-p53 immunoblotting. Quantification of the levels of pY-p53 relative to the untreated cells are indicated, and were normalized to total p53 levels. d Schematic representation of p53 domain organization highlighting the location of Y327 with the relevant domains indicated. e Radioactive in vitro kinase assay performed using purified FLAG-NEK10 WT and the indicated GST-p53 constructs. f Quantification of relative phosphate transfer (p < 0.05, two tailed t-test, n = 3 technical replicates, bars represent SEM). g Indicated p53 constructs were transfected into NEK10WT and NEK10Δ44 A549 cells and the phosphotyrosine-containing proteins were immunoprecipitated. The amount of pY-p53 was determined by anti-p53 immunoblotting.

we assessed in breast cancer datasets (METABRIC, with overall survival on p53 Y327. Exposure of cells to IR also prompted tyrosine phosphorylation of WT p53, which was abolished by p53 Y327F substitution (Fig. 6e). Supporting the importance of Y327 phosphorylation for the p53 transcriptional response, reconstitution of H1299 cells (p53-null) with the p53 Y327F mutant failed to fully support expression of p21 following IR, when compared with the same cells expressing WT p53 (Fig. 6f).

We next probed the effect of NEK10 status on cellular sensitivity to escalating doses of genotoxic agents using clonogenic assays. NEK10 loss led to increased cell sensitivity to cisplatin in both A549 and MCF-7 cells (Fig. 7a, b), an effect paralleled by the impact of NEK10 loss on DNA damage-induced cell cycle arrest. In response to cisplatin treatment, and in contrast to NEK10WT cells, NEK10Δ44 cells were compromised in the induction of the G2/M arrest (Fig. 7c, d). The G2/M arrest defect in NEK10Δ44 cells was accompanied by heightened cell death, evidenced by the increased proportion of both the Sub-G1 and Annexin V+/PI+ cells, (Fig. 7e, f).

NEK10 expression levels are predictive of wildtype TP53 breast cancer outcome

Based on previous evidence of genetic alterations in and around the NEK10 gene in breast cancers [22–25, 27, 40, 41], and the newly found function of NEK10 in p53 regulation, we assessed influence of the functional NEK10-p53 relationship on outcome in two breast cancer datasets (METABRIC, with overall survival (OS) as outcome [42]) and The Cancer Genome Atlas (TCGA BRCA) (with progression-free survival (PFI) as outcome [43]). In contrast to patients with WT TP53 and low NEK10 expression, WT TP53 and high NEK10 expression was predictive of better outcome (Fig. 8a), an effect not seen in patients with somatic TP53 mutations (Fig. 8b).

To better understand the interaction(s) between NEK10 and p53 transcriptional activity in patient samples, we developed a p53-targets curated signature score (p53TCSS), using a list of high confidence p53-target genes [44], calculated using single-sample Gene Set Expression Analysis (ssGSEA). The p53TCSS paralleled the TP53 mutational status in both METABRIC and TCGA Breast datasets, with WT TP53 tumors displaying higher average p53TCSS when compared with mutant TP53 tumors (Fig. S11), thus validating p53TCSS as a surrogate for p53 transcriptional activity within these patient cohorts.

To determine if NEK10 expression status in combination with p53TCSS had an impact on patient survival, tumors from each patient cohort considered wildtype for TP53 were split into four groups according to high or low NEK10 expression, and to high or low p53TCSS (Fig. 8c). In both datasets, tumors with high NEK10/p53TCSS associated with better prognosis, while worse prognosis separated with low NEK10/p53TCSS (METABRIC p = 0.001, TCGA BRCA p = 0.022).

Radiotherapy is often administered as a means to prevent breast cancer recurrence, leading to improved prognosis and outcome [45, 46]. In the TCGA BRCA dataset, patients with low NEK10 expression who had received radiation therapy, compared with those that had not, had improved PFI (p = 0.045) (Fig. 8d). In contrast, there was no additive benefit of radiation therapy in the High NEK10 patient subset (Fig. 8d). Thus, genetic associations between NEK10 and TP53 parallel the functional interactions identified in cancer cell lines.

Discussion

The cellular response to DNA damage is a well-orchestrated sequence of molecular events within a complex network of intersecting signaling pathways. The process is modulated in a context-dependent manner and is sensitive to the phase of the cell cycle in which the damage is encountered, the type/degree of the damage, the extracellular milieu, or the mutational status of the cell itself. Central to many of the cellular damage response pathways is the tumor suppressor p53 [2, 47, 48]. The work presented here uncovers a function for the protein kinase NEK10 in regulating p53 transcriptional activity through phosphorylation of p53 Y327.
Loss of NEK10 increased cellular proliferation and DNA replication (Fig. 1) unlike that of other NEK family kinases, including close NEK10 homologues NEK6 and NEK7, whose loss of function leads to a decrease in cellular proliferation or induction of cell death [49–51]. The mechanism behind the NEK10-dependent effect on cell proliferation related to a decrease in the expression of p53-responsive genes, key regulators of cell cycle progression (Fig. 2a, b) [30, 31]. The effect was largely dependent on p53, as knockdown experiments revealed a synergistic relationship between NEK10 and p53 on the expression of p53-target genes (Fig. 2c). Of note, NEK10 may also contribute to the expression of p53-responsive genes in a p53-independent manner.
manner, as overexpression of NEK10 in shp53 NEK10-deficient A549 cells still led to a slight increase in p21 expression (Fig. 3e).

Although NEK10 loss led to a decrease in p53 protein in A549 cells, possibly via the auto-regulatory p53 response element within the human TP53 promoter, acute re-expression...
Fig. 7 NEK10 loss sensitizes cells to chemotherapeutic agents. 

a–b NEK10+/+ and NEK10Δ/Δ A549 and MCF-7 cells were treated with the indicated doses of cisplatin and assessed for clonogenic survival 10–12 days after treatment. Graph represents proportion of cells (using a logarithmic scale) that survive after treatment compared with untreated control, (n = 3 biological replicates, p < 0.05, two tailed t-test, bars represent SEM).

c) Cells were treated with 5 μM of cisplatin overnight and allowed to recover for 24 h prior to being analyzed for DNA content with propidium iodide.

d) Quantification of G2 populations for cisplatin treated cells as an indicator of G2/M arrest (p < 0.01, two tailed t-test, n = 3 biological replicates, bars represent SEM).

e) Cells were treated with 5 μM cisplatin for 20 h and then assessed for proportion of sub-G1 cells, an indicator of cell death, at the indicated time points after treatment (p < 0.05, two tailed t-test, n = 3 biological replicates, bars represent SEM).

f) Cells were treated with indicated doses of cisplatin and assessed for apoptosis with Annexin V/propidium iodide staining 24 h after overnight cisplatin treatment (p < 0.05, two tailed t-test, n = 3 biological replicates, bars represent SEM).
of NEK10 did not fully restore p53 protein levels (Figs. 2d, 3c–e) [52–54]. Despite this, NEK10 re-expression did increase the levels of p21 in a mostly p53-dependent manner, indicating that NEK10 loss over multiple passages may have an effect on p53 levels not captured in shorter term re-expression experiments (Fig. 3c–e).

The differences in p53-target gene expression were predominantly dependent on the tyrosine kinase activity of NEK10 (Fig. 3c, d), as the serine phosphorylation-restricted NEK10 mutant (I693P) (Fig. S7) failed to augment p53-target gene expression in NEK10Δ/Δ cells (Figs. 3c, d and 5f) [36]. While re-expression of both Nek10 kinase-defective variants failed to rescue expression of p53-responsive genes, they led to equivalent increases in MDM2 protein levels (Fig. 3c, d), as the serine phosphorylation-restricted NEK10 mutant (I693P) (Fig. S7) failed to augment p53-target gene expression in NEK10Δ/Δ cells (Figs. 3c, d and 5f) [36]. While re-expression of both Nek10 kinase-defective

variants failed to rescue expression of p53-responsive genes, they led to equivalent increases in MDM2 protein levels (Fig. 3c, d), suggestive of a possible kinase-independent effect of NEK10 on the MDM2 protein, possibly explaining their mild, but quantifiable effect on cellular proliferation (Fig. 3a).

Reconstitution of p53-dependent transcription, or that driven by its non-phosphorylatable p53 Y327F mutant in HCT116 p53−/− cells, revealed Y327 phosphorylation as a separate threshold for transactivation of p53-targets (Fig. 5). Namely, p53 Y327F induced an intermediate level of p53-target gene expression when compared with WT p53 and the loss of function p53 L344P mutant, reflective of a partial loss of p53 function in NEK10-deficient cells (Figs. 2a, b, 5a, b). Only in cells expressing WT p53, overexpression of tyrosine kinase-competent NEK10 impacted transactivation of the p53-target genes p21 and MDM2 (Fig. 5d–f, S9). The Y327F p53 appears to act as a hypomorph, driving a phenotype also found in NEK10-deficient cells, specifically a differential reaction to DNA-damaging agents (Figs. 6, 7, S10), such as the protracted kinetics of p53 and p21 induction in response to IR (peaking at 4 h), compared with a rapid induction and an early plateau in NEK10-proficient cells (0.5–2 h) (Fig. 6c, d). While the changes in p53 tyrosine phosphorylation paralleled those in p53 S15 phosphorylation and an increase in p53 transcriptional activity, p53 Y327F mutation or NEK10 loss did not affect p53 S15 phosphorylation. (Fig. 6a, c, d, f).

The weakened p53 response in NEK10Δ/Δ cells may contribute to their sensitivity to cisplatin, presenting as increased apoptosis and decreased clonogenic survival (Fig. 7a, b). NEK10Δ/Δ cells failed to engage an effective G2/M arrest in response to cisplatin possibly due to the lack of induction of GADD45α, a key mediator of the G2/M checkpoint, (Figs. 6a, b, 7c, d, S10) [55–57]. Of note, despite lowered expression of the pro-apoptotic p53-responsive genes BAX and PUMA, NEK10-deficient A549

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cells were more sensitive to cisplatin, displaying increased cell death (Figs. 2b, 6b, 7e, f, S10). Thus, NEK10-dependent changes in cell cycle arrest and death may reflect a selective, prioritized response to genotoxic stressors, whereby wild-type NEK10 supports cell cycle arrest through p53 phosphorylation, whereas in the absence of NEK10, the balance shifts towards p53-dependent apoptosis, due to accrual of deleterious DNA lesions. Perhaps Y327 p53 phosphorylation works in concert with other posttranslational modifications to fine tune the p53 response towards cell cycle arrest, similar to the PCAF-mediated p53 Lys320 acetylation [10, 58–60].

Consistent with the diminution of p53 function in NEK10-deficient cells, analysis of genome data revealed associations of NEK10 and p53 status in breast cancers, further suggestive of a tumor-suppressive function of NEK10, via its impact on p53 (Fig. 8) [22–25, 27]. NEK10 expression correlated with better disease outcome in WT TP53 breast cancers (Fig. 8a, b). While TP53 loss of function is predictive of treatment success in certain breast and other cancers, p53 transcriptional activity has largely been understudied as a prognostic cancer marker. A curated signature of p53-target gene expression (p53TCSS) developed here, as a surrogate for p53 transactivation activity in cancers (Fig. S11), in combination with existing markers, may have utility as a prognostic biomarker for the already standard of care chemo/radio-combinatorial score [42, 61–64].

Materials and methods

Antibodies

Anti-GAPDH FL-335 (sc-25778), Anti-p53 FL-393 (sc6243), Anti-p-Tyr PY-99 (sc-7020), Anti-GADD45α C-4 (sc-6580) (Santa Cruz Biotechnology). Anti-FLAG M2 (F3165) and Anti-FLAG M2 Agarose (Sigma-Aldrich). Anti-p21 (556431) (BD Pharmingen). Anti-MDM2 Ab-1 (OP46) (Calbiochem). Anti-PUMA (NB500-261) (Novus Biologicals). Anti-p53 S15 (9284) and Anti-MDM2 (OP46) (Calbiochem). Anti-PUMA (NB500-261) (Novus Biologicals). Anti-p21 (556431) (BD Pharmingen). Anti-MDM2 Ab-1 (OP46) (Calbiochem). Anti-PUMA (NB500-261) (Novus Biologicals). Anti-p53 S15 (9284) and Anti-MDM2 (OP46) (Calbiochem). Anti-FLAG M2 (F3165) and Anti-FLAG M2 Agarose (Sigma-Aldrich). Anti-p21 (556431) (BD Pharmingen). Anti-MDM2 Ab-1 (OP46) (Calbiochem). Anti-PUMA (NB500-261) (Novus Biologicals).

Plasmids

For mammalian cell expression, NEK10 was cloned into 3xFLAG-CMV-7.1 or pLVX, and p53 was cloned into pcDNA3-His. For bacterial expression, p53 was cloned into pGEX2TK. Point mutants were generated by QuickChange site-directed mutagenesis kit (Stratagene). pSpCas9n(BB)-2A-Puro (pX462) V2.0 was a gift from Feng Zhang (Addgene plasmid 62987).

Cell culture

HEK293T, A549, MCF-7, H1299, HCT116 cells, and their derivatives were maintained in DMEM (Corning), supplemented with 10% FBS (Wisent) and Pen/Strep (100 μg/ml, Hyclone).

HEK293T cells were transfected by the calcium phosphate method, other cell lines with PolyJet™ (FroggaBio). NEK10ΔΔ cells were generated by targeted deletion of NEK10 exon 24, using paired sgRNA-guided Cas9 nickases (described in Fig. S1). sgRNAs were cloned into pX462, transfected into cells and puromycin selected (0.5 μg/ml) for 48 h, prior to isolation and expansion of clones.

A549 NEK10ΔΔ cells were engineered to express indicated pLVX plasmids via lentiviral transduction in the presence of protamine sulfate (5 μg/ml) for 20 h, reseeded into 200 μg/ml hygromycin (Sigma) and selected until negative control cells died (3–5 days).

Cell lysis, immunoblotting, and immunoprecipitations

Unless indicated otherwise, for immunoblotting, cells were lysed in Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol), normalized for total protein, resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore).Membranes were blocked in 5% BSA and probed with the indicated antibodies.

For denaturing immunoprecipitations, cells were lysed as described above and sonicated on ice, clarified by centrifugation at 15,000 × g for 15 min. Equalized lysates were diluted in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 0.1 mM Na3VO4 and protease inhibitor cocktail (Sigma)). Immunoprecipitations were performed by end-over-end 3 h rotation with indicated antibodies, incubated with protein A/G-sepharose beads (45°), washed four times in RIPA buffer and resuspended in Laemmli loading buffer.

Protein half-life determination

Cells were incubated with 200 μg/ml of cyclohexamide (Sigma-Aldrich) for the indicated times and lysed in Laemmli loading buffer. Protein levels were detected by immunoblotting.
Proliferation and clonogenic assays

Cell numbers were assessed indirectly by sulforhodamine B (SRB) assay as described in [65].

Clonogenic assays were performed by seeding 100–1000 cells/well in a 6-well plate, treating as indicated and scored at 14–16 days. Cells were fixed and dyed as described in [66]. Colonies were counted in ImageJ with the CellCounter plugin.

Quantitative reverse transcription PCR (RT-qPCR) analysis

Total RNA was isolated by EZ-10 DNAaway RNA Mini-prep kit (Bio Basic). Reverse transcription was performed using the qScript cDNA SuperMix (Quantabio). RT-qPCR analysis was performed using PerfeCTa SYBR Supermix with 20–50 ng of cDNA per reaction and 200 nM of gene-specific primers (listed in Table S1).

qPCR was performed on the Roche LightCycler 480, and relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference [67].

Flow cytometry cell sorting (FACS) analysis

All FACS was performed on FACSCanto flow cytometer using CellQuest software (BD Biosciences) and analyzed by FlowJo.

For both methods of cell cycle analysis, cells were seeded into 6 cm plates, treated as indicated, trypsinized, and fixed for 1 h in cold 70% ethanol.

For total DNA content analysis, washed cells were stained with 50 μg/mL PI, 20 μg/mL RNase A, 0.1% Triton X-100 and analyzed by FACS.

For quantification of mitotic cells, ethanol fixed/0.25% Triton X-100 permeabilised cells were stained with 0.25 μg anti-phospho-H3-S10 (Millipore) followed by visualization with Alexa488-conjugated goat anti-mouse IgG, and counterstaining (50 μg/mL PI) followed by FACS analysis.

Proportion of cells with active DNA synthesis was determined by a modified EdU pulse labeling protocol that utilizes Click chemistry as previously described [68] and quantified by FACS.

Apoptosis was quantified by Annexin V/PI assays by flow cytometry according to the manufacturer’s instructions (BD Pharmingen).

Protein purification

GST-p53 and GST-p53 Y327F were produced in BL21 E. coli cells from single colonies and expression induced at OD600 = 0.5 with 0.5 mM of IPTG for 4 h. Snap-frozen cell pellets were lysed in PBS/1% Triton X-100/lysozyme and sonicated (3x). GST-fusion proteins were purified from clarified lysates with 20 μL/mL of glutathione sepharose 4B resin, washed, and eluted with 10 mM reduced glutathione, 50 mM Tris-HCl pH 7.6, 150 mM NaCl. Protein purity and quantity were determined via SDS-PAGE Coomassie staining.

In Vitro kinase assays

Kinase assays were performed by the incubation of 100–200 ng of purified NEK10 at 30 °C for 30 min in kinase assay buffer supplemented with 5 μCi [γ-32P] ATP, 20 μM ATP and 1 μg of GST-p53 or GST-p53 Y327F, reactions terminated using boiling Laemmli loading buffer, resolved by SDS-PAGE, imaged on Typhoon Imager (GE Healthcare Lifesciences) and ImageJ quantified.

Oligomerization assay

Cells were treated as indicated and lysed with a buffer containing: 25 mM Hepes pH 7.5, 150 mM NaCl, and 1% NP-40, supplemented with 1 mM DTT, 0.1 mM Na3VO4 and protease inhibitor cocktail (Sigma). Cleared, equalized lysates were crosslinked with 0.025% Glutaraldehyde and the reaction quenched with 100 mM Glycine pH 2.5.

Bioinformatics

METABRIC and TCGA clinical and normalized gene expression data were obtained from cBioPortal for Cancer Genomics (http://www.cbioportal.org/publicportal/) and from the corresponding publications [42, 43, 69]. Somatic mutation and genetic variation data was obtained following approval by the dbGaP Data Access Committee (project #11689). Multivariate Cox regression analyses using R software (survfit package) included age at diagnosis, tumor stage, and histology as covariates. When appropriate, the follow-up were censored at 10 years. The p53-targets curated signature score (p53TCSS) was obtained from a list of high confidence p53 targets [44] and scores computed using the ssGSEA with the GSVA package in Bioconductor [70]. The significance of gene expression correlations was determined computing PCCs and t-tests using R software. Gene expression differences between TP53 WT and mutant tumors was determined by computing logistic regressions with age, tumor stage, and histology as covariates.

Statistical analyses

Data are presented as the Mean ± S.E.M of independent biological experiments, unless otherwise specified. The P values, replicate number (at least $n = 3$), type of experimental replicate, and statistical test are indicated in the
figure legends. Appropriate statistical analyses were performed with GraphPad Prism 7.0, using two-tailed Student’s t test for 2-group comparison, unless otherwise specified in the figure legends. A P value of <0.05 was considered to be significant. Data largely met the assumptions of the indicated statistical tests.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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