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IPH-926 lobular breast cancer cells harbor a p53 mutant with temperature-sensitive functional activity and allow for profiling of p53-responsive genes

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Profiling of p53-responsive genes has been carried out in different cellular models, most of which involved genetic modifications or cytotoxic stimulation. We report on the utilization of IPH-926 human lobular breast cancer cells for the profiling of p53-responsive genes using a novel approach without such modifications. We discovered that IPH-926 cells harbor a homozygous *TP53* missense mutation encoding for a rare p53 mutant (E285K) with temperature-sensitive (ts) loss of function characteristics. This mutation had evolved as a late, secondary genetic event during the natural clonal evolution of the corresponding lobular carcinoma. *In vitro* temperature shifts reconstituted endogenous wild-type p53 activity in IPH-926, as evidenced by induction of $p21^{Waf1}$. Transcriptional alterations associated with restored p53 function were profiled using Affymetrix microarrays and a new strategy to gate out non-specific temperature effects. At the $P = 0.0005$ significance level, 60 genes were differentially expressed following reconstitution of p53 activity. These genes included *CDKN1A, MDM2* and *PHLDA3*, a recently described p53-inducible inhibitor of AKT. Similar transcriptional alterations were observed upon reconstitution of p53 activity in BT-474 cells, which also harbor ts-p53 E285K, and in ASPC1 cells transduced with ts-p53 A138V. Consistent with these models, low *PHLDA3* expression was associated with nuclear p53 accumulation, indicative of deleterious *TP53* mutations, in primary breast cancers. From a molecular point of view, IPH-926 thus provides a new tool to study transcriptional programs controlled by p53. From a tumor pathology perspective, IPH-926 also provides the first direct evidence of a p53-related clonal evolutionary pathway in lobular breast cancer progression.

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Mutational inactivation of the *TP53* tumor suppressor gene encoding for p53 is frequent in human breast cancer, especially in estrogen receptor (ER)-negative, medullary, basal-like and *BRCA1*-related cases.[1–4] The p53 protein functions as a stress-induced transcription factor that regulates various cellular processes, such as growth arrest, apoptosis and energy metabolism.[5] External stresses that stabilize p53, and thus induce p53 signaling, include DNA-damaging agents and hypoxia.[6] Internal stresses that induce p53 signaling include activation of oncogenes, such as *PIK3CA* and *PTEN*.[7] Characterization of p53 transcriptional targets is of importance for understanding how mutational inactivation of *TP53* promotes tumor initiation and progression. Profiling of p53-responsive genes has been carried out in different cellular models. Some of these models relied on cytotoxic cell stimulation for induction of a p53 response.[8,9] Other models utilized genetically engineered cells, which overexpressed wild-type (wt) p53, *TP53* targeting shRNAs for p53 knockdown, or p53 inactivating polypeptides for functional neutralization of p53.[10–16] Another useful approach has been ectopic overexpression of the murine, temperature-sensitive (ts) p53 missense mutant A135V. The temperature-sensitive loss of function characteristics of ts-p53 A135V were discovered by chance owing to a misadjusted cell culture incubator.[17] This mutant displays wt p53 activity at about 32 °C (permissive condition) but loses this activity at about 37 °C and above (restrictive condition) due to an instability of the correct protein folding at body temperature.[18]

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Its human homolog, ts-p53 A138V, was generated by site-directed mutagenesis and showed the same functional characteristics.[19] According to the IARC *TP53* database, ts-p53 A138V is very rare in real human tumors (approximately one in 500 *TP53* mutations).[20] Nonetheless, the ts-p53 A138V clone generated by Hirano and colleagues has become one of the most widely used constructs in p53 research. Various p53-null cell lines have been genetically modified to overexpress ts-p53 A138V. Subsequent comparison of global gene expression at restrictive *vs* permissive condition uncovered novel p53 target genes, such as *DDB2*.[21–24] Of note, one features is common to all of the above-cited strategies for the profiling of p53-responsive genes. To turn p53 activity on and off, the above-cited models required substantial manipulation of the cells being investigated. Depending on the experimental approach, these manipulations included viral infection or selection processes for ectopic overexpression of appropriate constructs or direct cytotoxic stimulation with DNA-damaging agents. Meanwhile, thousands of recombinant p53 mutants, each harboring a unique amino-acid substitution, have been functionally characterized to assist the interpretation of *TP53* missense mutations in clinical tumor specimens.[25] These analyses revealed another 142 p53 mutants with temperature-sensitive loss of function characteristics.[26] The newly discovered ts-p53 mutants are also rare in real human tumors, but E285K is the most common one (approximately one in 150 *TP53* mutations).[27] Ts-p53 E285K displays a strictly temperature-dependent loss of function, as determined by reporter gene assays using p53 DNA-binding sites of various classical p53 target genes, such as $p21^{Waf1}$/*CDKN1A* and *MDM2*.[26,27] In the present study, we employed a new experimental approach for the profiling of p53-responsive genes, which circumvents genetic modifications and cytotoxic stimulation. This model is based on IPH-926, a human infiltrating lobular breast cancer (ILC) cell line established recently in our laboratory and BT-474, a human infiltrating ductal breast cancer (IDC) cell line established in 1976.[28–30] Here, we report that both cell lines harbor an endogenous ts-p53 E285K mutant, which facilitates reconstitution of endogenous wt p53 activity and assessment of p53-responsive genes. In particular, we describe the discovery of the *TP53* E285K mutation in IPH-926, show that *in vitro* temperature shifts reconstitute their endogenous wt p53 activity, as evidenced by induction of $p21^{Waf1}$, and report the profiling of transcriptional alterations associated with restored p53 function using Affymetrix microarrays. In addition to the new experimental approach of this study, namely the use of breast cancer cells harboring endogenous ts-p53 for profiling of p53-regulated transcripts, our findings also provide insight into ILC progression. ILC is a biologically distinct tumor entity, which is characterized by an indolent clinical behavior and inactivation of the *CDH1* tumor suppressor gene encoding for E-cadherin.[31,32] *TP53* mutations are very rare in primary ILCs.[1,4] However, it has been speculated that acquisition of *TP53* mutations may drive late stage ILC progression or development of so-called pleomorphic ILCs (PLCs), which are very aggressive.[33–39] In line with this assumption, we also demonstrate by direct sequencing of related clinical tumor specimens that the ts-p53 E285K mutation in IPH-926 ILC cells had evolved as a late, secondary genetic event during the natural clonal evolution of the corresponding lobular carcinoma. IPH-926 ILC cells are thus not only a new tool to study p53 function but also provide the first direct evidence for the involvement of p53 in human ILC progression.

MATERIALS AND METHODS

Cell Culture

Human breast cancer cell lines used in this study have been described previously.[28,30] All cell lines were authenticated by short tandem repeat (STR) profiling. IPH-926 cells were additionally authenticated by PCR-based detection of the unique *CDH1* 241ins4 mutation.[28] As described previously, IPH-926 had been derived by clonal isolation of an epitheloid colony identified in malignant ascites subjected to short-term cultivation in $6 \times 140\,mm^2$ (diameter) dishes holding serum-free DMEM/F12 medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10 μg/ml bovine insulin and 0.1 mM non-essential amino acids.[28] Isolated cells had then been expanded in RPMI-1640 containing 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 2.5 g/l glucose, 10 μg/ml bovine insulin and 20% fetal calf serum (FCS).[28] For the experiments described herein and for routine cultivation of IPH-926, FCS was reduced to 10%. IPH-926 xenograft tumors were generated in female NOD/SCID mice as described previously.[28] ASPC1 p53-null carcinoma cells retrovirally transduced with ts-p53 A138V were kindly provided by D Nuevemann and H Ungefroren.[40] All cells were kept in a water-saturated atmosphere containing 5% CO_2 at 37.5 °C (restrictive condition) or 32.5 °C (permissive condition). Roscovitine was purchased from Sigma (Deisenhofen, Germany).

Tumor Specimens

Formalin-fixed paraffin-embedded (FFPE) primary breast cancer specimens ($n = 46$) were retrieved from the tissue archive of the Hannover Medical School according to the guidelines of the local ethics committee. All specimens were made anonymous for scientific purposes and were compiled on tissue microarrays for immunohistochemical assessment of nuclear p53 accumulation as described previously.[41] Clinicopathological characteristics are reported in Supplementary Table 1.

DNA Isolation and Genetic Analyses

Genomic DNA was isolated from cell lines and FFPE specimens using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). DNA concentration was determined with a NanoDrop 2000 spectrophotometer (NanoDrop Products, Wilmington, USA). Genetic analysis of the *TP53* gene was performed by PCR and direct sequencing of exons 4 to 9,

which cover the *TP53* mutational hot spot regions.[42] Each exon was amplified individually using 10 ng DNA as template. Next, ~50 ng of purified PCR product (Qiaquick kit, Qiagen) were directly subjected to dye-terminator sequencing. Sequence data were analyzed with SeqMan software and GeneBank nucleotide NM_000546 as a reference. For detection of loss of heterozygosity (LOH) at the *TP53* locus, tumor cells were purified from FFPE tissue blocks by laser-capture micro-dissection (PALM Laser-MicroBeam System, Zeiss, Jena, Germany) followed by isolation of genomic DNA as described previously.[43] Next, the polymorphic dinucleotide marker TP53CA was amplified by PCR and fluorescence-labeled PCR products were detected by capillary gel electrophoresis on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Forster City, USA).[44]

Microarray Analysis

Microarray expression analyses were carried out as described previously.[45] In brief, cells were lysed in RLT buffer (Qiagen) and were subjected to extraction of total RNA. RNA amplification and labeling was done according to the manufacturer's protocol (Small Sample Target Labeling Assay Version II, Affymetrix; Santa Clara, USA) and 10 μg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing biotinylated hybridization controls (BioB, BioC, BioD, Cre). All samples were hybridized to an identical lot of Affymetrix HG U133 Plus 2.0 GeneChips for 16 h at 46 °C. Subsequently, GeneChips were washed, stained with SA-PE and read using an Affymetrix GeneChip fluidic station 400 and a GCS 3000 scanner. Raw data have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE35006). Probe set signal log values (SLV) were extracted, normalized and $\log_2$-transformed using the RMA algorithm and Expression Console™ software.[46] Next, expression data from IPH-926 were uploaded into the statistical package BRB-ArrayTools version 3.5.0-Patch_2 for filtering and statistical analyses. Along with the data from IPH-926 cells (harboring ts-p53 E285K), we uploaded expression data from MCF-7 cells (harboring wt p53). This served to gate out non-specific temperature effects as outlined below. First, probe sets with low signal intensity (<100) were excluded. Next, two-class comparison was performed to define genes differentially expressed between MCF-7 cells at restrictive *vs* permissive condition using parametric *t* tests and a $P=0.001$ significance threshold. Identified probe sets were considered as non-specifically regulated by temperature. These probe sets were excluded from subsequent analysis of IPH-926 expression data (Figure 5a). Next, two-class comparison was performed to identify genes differentially expressed between IPH-926 cells at restrictive *vs* permissive condition using parametric *t* tests, a stringent significance threshold of $P=0.0005$, and the cleared probe set population (Figure 5a). Identified transcripts were considered to represent candidate p53-responsive genes in IPH-926. Finally, these analyses were repeated in a second BRB-ArrayTools project, which included expression data from BT-474 cells (also harboring ts-p53 E285K) and MCF-7 cells (harboring wt p53).

Quantitative Real-Time RT–PCR

Extraction of total RNA and cDNA synthesis were performed as described previously.[28] Quantitative assessment of gene expression normalized to two housekeeping genes (*β-GUS* and *TBP*) was performed with Platinum Taq DNA polymerase (Invitrogen), Sybr Green I (Invitrogen) and QuantiTect® primer assays (Qiagen) on an ABI Prism 7700 system (Applied Biosystems, Foster City, USA). For *in vitro* experiments, statistical significance was determined by parametric *t* tests using GraphPad Prism software. For primary mammary carcinomas, significance of associations of clinicopathological characteristics with low/absent *vs* moderate/high *PHLDA3* expression was determined by χ^2 tests.

Western Blot

Cells were lysed in RIPA buffer and 40 μg total cellular protein were separated by 12% SDS–PAGE and were transferred to nitrocellulose membranes. Membranes were probed with anti-p53 (clone DO-1, Novocastra Laboratories, Newcastle, UK), anti-$p21^{Waf1}$ (clone 70, BD Transduction Laboratories, Franklin Lakes, USA), anti-PARP (clone C-2-10, Calbiochem, Darmstadt, Germany) and anti-β-actin antibodies (clone AC15, Acris, Hiddenhausen, Germany).

Immunohistochemistry

Nuclear p53 accumulation was determined by immunohistochemical staining of FFPE breast cancer cell lines and human primary breast cancers compiled on tissue microarrays using the monoclonal anti-p53 antibody DO-7 (Novocastra) diluted 1:100. The epitope detected by the DO-7 antibody corresponds to p53 amino-acid residues 1–45, which are generally not affected by missense mutations.[42,47] Classical deleterious missense mutants of the p53 protein, which mostly affect the amino-acid residues 126–307, are associated with altered protein conformation and prolonged protein half-life.[42,47,48] These p53 mutants are indirectly detectable with the DO-7 antibody through demonstration of an aberrant nuclear p53 accumulation.[42,47,48] Detection of immune reactions was achieved with the ZytoChem-Plus HRP Kit (Zytomed, Berlin, Germany). The cutoff for the definition of a nuclear p53 accumulation was $\geq$90% tumor cells showing a very strong stain intensity (scored 3 on a scale of 0–3). The cutoffs for the Ki67 labeling index (LI) used to separate tumors with low *vs* moderate or moderate *vs* high proliferation were 10 and 25%, as described previously.[49]

Cell Viability Assays

Relative cell viability was determined with the WST-1 assay (Roche, Mannheim, Germany) in 96-well culture plates according to the manufacturer's recommendations. Before enzymatic readout reactions were started, all plates were

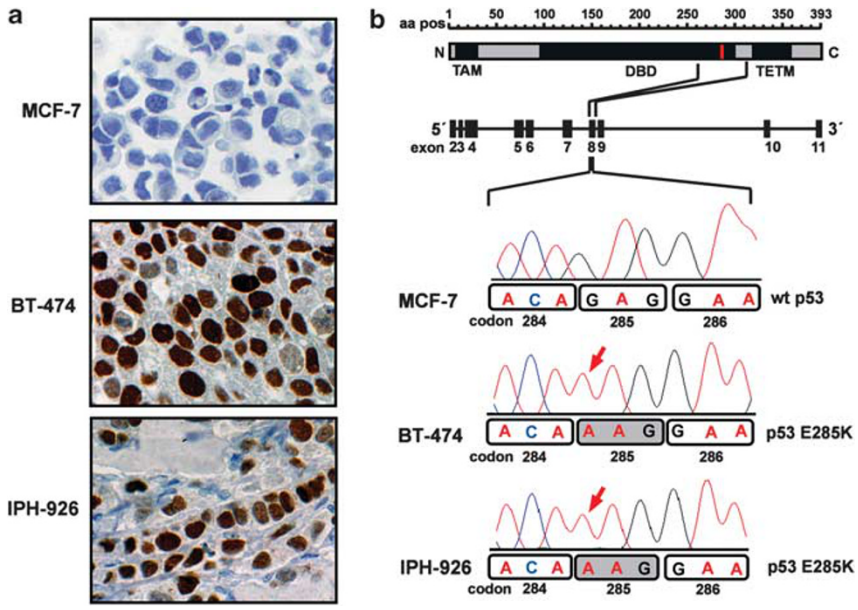

Figure 1 Identification of a *TP53* mutation in IPH-926. (**a**) Immunohistochemical detection of nuclear p53 accumulation (magnification, $\times 400$). The photomicrograph corresponding to IPH-926 was taken from a NOD/SCID mouse xenograft tumor.[28] IPH-926 cells cultured *in vitro* at regular conditions (water-saturated atmosphere containing 5% CO_2 at 37.0–37.5 °C) displayed the same nuclear p53 accumulation. (**b**) Detection of a homozygous *TP53* missense mutation in IPH-926 and BT-474. The upper panel shows the p53 protein with key functional domains and the genomic organization of the *TP53* exons (TAM; transactivation motif, DBD; DNA-binding domain, TETM; tetramerization motif, aa pos; amino-acid position). The missense mutation detected in IPH-926 and BT474 is located in exon 8, which encodes for the C-terminal part of the DNA-binding domain and results in an E285K amino-acid substitution.

readjusted to 37.5 °C culture temperature. PARP cleavage, indicative of apoptotic cell death, was assessed by western blot as described previously.[28] For cell-cycle analysis, cells were fixed in 70% ice-cold ethanol and were subjected to RNAase A treatment (50 μg/ml; Roche). Cells were stained in PBS/2 mM EDTA/0.1% Triton-X-100 containing 20 μg/ml propidium iodide (Sigma, Germany) 30 min before analysis on FACS Calibur flow cytometer.

RESULTS

Identification of an Endogenous *TP53* Mutation in IPH-926 ILC Cells

IPH-926 is a recently established human ILC cell line.[28,29] IPH-926 facilitates *in vitro* analyses of human ILC cells, for which a well-characterized model had not been available before.[28] Interestingly, IPH-926 showed a nuclear accumulation of p53 at regular cell culture conditions (water-saturated atmosphere containing 5% CO_2 at 37.0–37.5 °C) and in NOD/SCID mouse xenograft tumors (Figure 1a). Nuclear p53 accumulation is indicative of *TP53* missense mutations.[4] This is due to impaired degradation of mutant p53 proteins. Although primary ILCs typically lack *TP53* alterations, we analyzed the *TP53* mutational status of IPH-926. This revealed a homozygous *TP53* missense mutation encoding for a mutant p53 protein (E285K) (Figure 1b). Consistent with previous reports, BT-474 ductal breast cancer cells harbored the same homozygous *TP53* missense mutation.[50,51] BT-474 cells also showed nuclear p53 accumulation (Figure 1a). In contrast, nuclear p53 accumulation was absent in MCF-7 ductal breast cancer cells harboring wt p53 (Figure 1). Hence, a *TP53* mutation was newly identified in IPH-926 ILC cells, which is an unusual aberration in this breast cancer entity.[1,4]

The *TP53* Mutation in IPH-926 ILC Cells Evolved as a Secondary Genetic Event

TP53 mutations may arise spontaneously during *in vitro* cultivation.[52] This may explain the higher frequency of *TP53* mutations in breast cancer cell lines compared with primary tumors.[51] As ILCs typically lack *TP53* alterations, we were interested in a retrospective evaluation of the *TP53* status of the original tumor specimens corresponding to IPH-926, which were available from institutional FFPE tissue archives. As reported previously, a unique somatic frameshift mutation of the *CDH1* tumor suppressor gene had been detected in the corresponding primary ILC, the corresponding locally recurrent ILC (developed 6 years after initial diagnosis), the corresponding distant dermal metastasis (developed 8 years after initial diagnosis) and in the IPH-926 cell line (derived from malignant ascites 16 years after initial diagnosis) (Table 1).[28] This had established the clonal relationship of these metachronous tumor specimens.[28] However, the primary ILC lacked nuclear p53 accumulation and showed a wt *TP53* sequence, as usually seen in classical ILCs. Yet, the corresponding locally recurrent ILC, which had developed 6 years after initial tumor diagnosis, harbored both, the *CDH1* frameshift mutation

Table 1 ***TP53*** **and** ***CDH1*****/E-cadherin status in archival clinical tumor specimens corresponding to IPH-926**

	Primary ILC	Local recurrence	Distant metastasis	IPH-926
CDH1/E-cadherin[a]				
Protein expression	Neg	Neg	Neg	Neg
LOH	nd	nd	Pos	Pos
Mutation (nucleotide)	241ins4	241ins4	241ins4	241ins4
Mutation (protein)	V82fsX93	V82fsX93	V82fsX93	V82fsX93
TP53				
Nuclear accumulation	Neg	Pos	Pos	Pos
LOH	nd	Pos	nd	Pos
Mutation (nucleotide)	wt	853G > A	853G > A	853G > A
Mutation (protein)	wt	E285K	E285K	E285K
Histological features[a]				
Single file linear cords	Pos	Pos	Pos	Pos
Solid growth	Neg	Pos	Pos	Pos
Granular cytoplasm	Neg	Neg	Neg	Neg
Histological grade	G1	G2	G3	ina
ER/PR/ErbB2 status	Pos/neg/neg	Neg/neg/neg	Neg/neg/neg	Neg/neg/neg

Neg, negative; pos, positive; nd, not determined; wt, wild type; ina, inapplicable.
[a]As reported previously.[28]

and the *TP53* missense mutation encoding for p53 E285K (Table 1). As described previously, the locally recurrent ILC had also acquired a higher histological grade through increased nuclear pleomorphism, a partially solid growth pattern and had lost estrogen receptor (ER) expression (Table 1).[28] Like mutation of *TP53*, these features are attributable to PLC.[33–39] Abundant granular cytoplasm, which is also occasionally associated with PLC, was not clearly evident (Supplementary Figure 1a).[34] Before the first occurrence of the *TP53* E285K mutation in the locally recurrent ILC, clinical treatment had included local irradiation and the selective estrogen response modifier tamoxifen, but no genotoxic chemotherapeutic agents (Supplementary Figure 1b).[28] The *TP53* E285K mutation in IPH-926 had thus evolved as a comparatively late, secondary genetic event during the *in vivo* clonal evolution of a classical ILC, which had acquired several features attributable to PLC.

Ts-p53 E285K Facilitates Reconstitution of Endogenous wt p53 Activity in IPH-926

According to analyses of recombinant proteins, p53 E285K is a ts-p53 mutant and its wt p53 activity is reconstituted at about 32 °C (permissive condition).[26,27] Cultivation of BT-474 cells at permissive condition restores their intrinsic wt p53 activity, as evidenced by induction of $p21^{Waf1}$ by ts-p53 E285K.[50] We sought to test whether this also applies to IPH-926 (Figure 2). BT-474 and IPH-926 cells, both harboring ts-p53 E285K, and MCF-7 cells, harboring wt p53, were cultivated at restrictive and permissive condition for 24 h. Subsequently, expression of *CDKN1A* mRNA encoding for $p21^{Waf1}$ was assessed by quantitative real-time RT–PCR. To demonstrate valid detection of *CDKN1A*, cells were also exposed to roscovitine, a chemical agent that stabilizes wt p53 and induces p53 signaling.[53] As expected, *CDKN1A* was upregulated by roscovitine in MCF-7 only, while permissive culture condition upregulated *CDKN1A* in BT-474 and IPH-926 only (Figure 2a). Consistently, $p21^{Waf1}$ protein was upregulated in BT-474 and IPH-926 cultured at permissive condition (Figure 2b). This upregulation of $p21^{Waf1}$ was accompanied by diminished expression of p53, which has previously been explained by restored negative auto-regulation of p53 (Figure 2b).[50] This view is compatible with the kinetics of *CDKN1A* upregulation in BT-474 and IPH-926. Immediately after the shift to permissive condition, *CDKN1A* expression increased 55-fold and 13-fold in BT-474 and IPH-926, respectively, reflecting an initial hyper-activation of wt p53 activity (Figure 3). After cells had adapted to cultivation at permissive condition for 24 h, *CDKN1A* mRNA expression leveled out at a plateau, which was three- to sixfold above baseline expression, indicating that a new equilibrium of wt p53 activity had been reached (Figure 3). *CDKN1A* expression remained at this plateau when BT-474 or IPH-926 cells were subjected to long-term cultivation at permissive condition (data no shown). Hence, ts-p53 E285K facilitates reconstitution of endogenous wt p53 activity in IPH-926 ILC cells through cultivation at permissive condition.

Reconstitution of Endogenous wt p53 Activity Fails to Induce Apoptosis in IPH-926

Enforced expression of wt p53 is harmful to cells and may induce growth arrest and apoptosis. We used the WST-1 assay, which reflects the number of metabolically active cells, to determine relative cell viability. Cultivation at permissive condition reduced viability in BT-474 and IPH-926 but not in MCF-7 cells, as determined by the WST-1 assay (Figure 4a). However, BT-474 and IPH-926 cell cultures were morphologically unaltered at permissive condition (Figure 4b). Consistently, we failed to detected PARP cleavage, indicative of apoptotic cell death, in any of the cell lines cultured at permissive condition (Figure 4c). In line with that, MCF-7, BT-474 and IPH-926 lacked apoptotic sub-G1 populations at permissive condition (Figure 4d). Of note, the vast majority of BT-474 and IPH-926 cells was in the G1 phase of the cell cycle at restrictive condition. At permissive condition, there was no further increase in the G1-phase populations (Figure 4d). Hence, BT-474 and IPH-926 cells experienced a metabolic suppression but did not undergo apoptotic cell death following reconstitution of endogenous

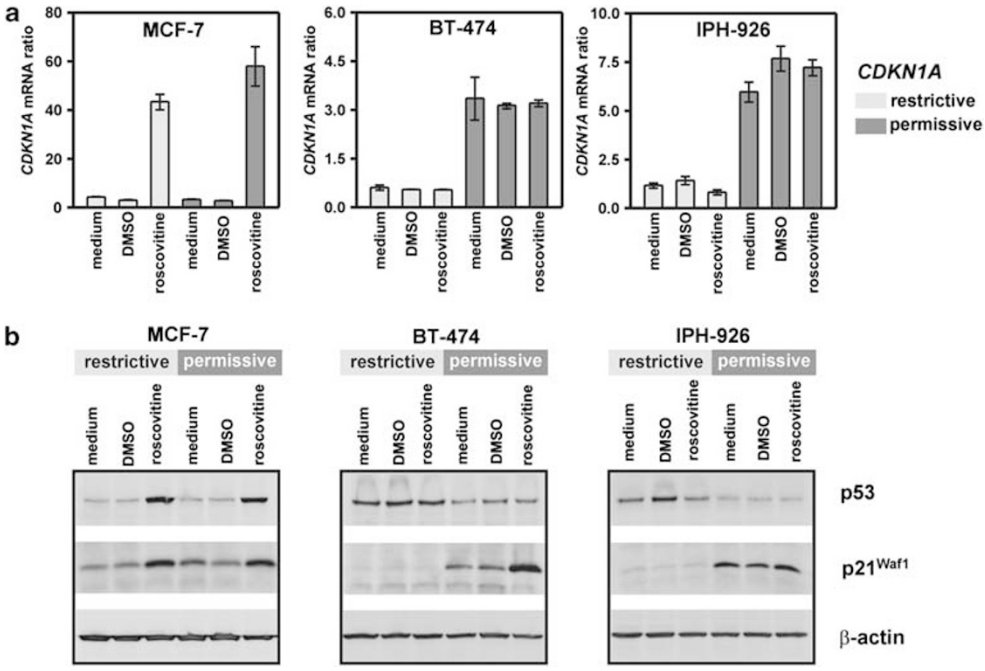

Figure 2 Upregulation of *CDKN1A* mRNA and p21^{Waf1} in IPH-926 and BT-474 at permissive condition. (**a**) Cells were cultured for 24 h at restrictive (37.5 °C) and permissive condition (32.5 °C) and *CDKN1A* mRNA expression was determined by quantitative real-time RT–PCR. Data are presented as mean mRNA ratio relative to two housekeeping genes. Error bars represent s.e.m. Exposure to roscovitine (20 μM) verified valid detection of p53-dependent upregulation of *CDKN1A* in MCF-7 harboring wt p53. Exposure to DMSO served as a solvent control for roscovitine. (**b**) Cells were cultured for 24 h at the conditions indicated. Total cellular protein was separated by 12% SDS–PAGE and was probed with anti-p53 and anti-p21^{Waf1} antibodies. Detection of β-actin verified equal loading.

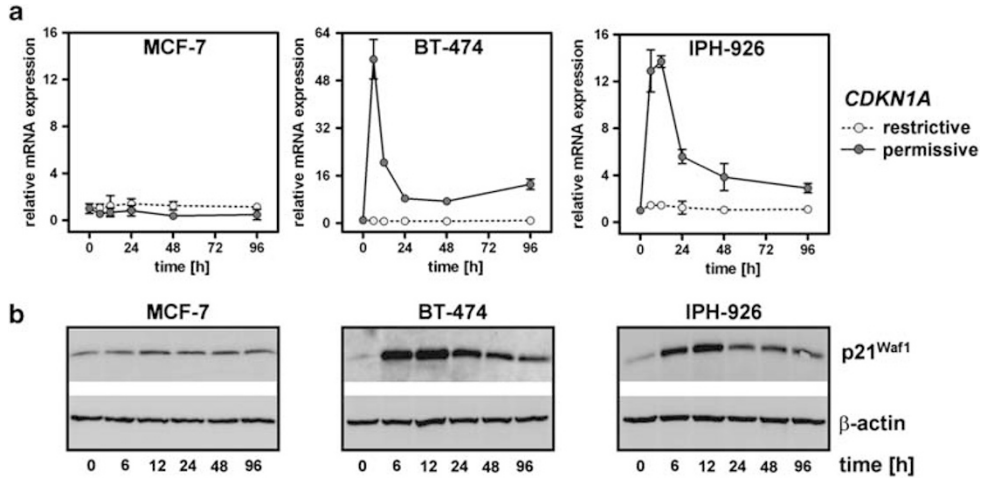

Figure 3 Kinetics of *CDKN1A* mRNA and p21^{Waf1} upregulation in IPH-926 and BT-474 shifted to permissive condition. (**a**) Cells were cultured at restrictive (37.5 °C) and permissive condition (32.5 °C) for different periods of time and *CDKN1A* mRNA expression was determined by quantitative real-time RT–PCR. Data are presented as mean relative expression with mRNA ratios of cells harvested at 0 h from dishes at restrictive condition adjusted to a value of 1. Error bars represent s.e.m. (**b**) Total cellular protein was separated by 12% SDS–PAGE and was probed with anti-p53 and anti-p21^{Waf1} antibodies. Detection of β-actin verified equal loading.

wt p53 activity, which facilitated their subsequent characterization under such conditions.

Definition of Transcriptional Alterations Associated with wt p53 Activity in IPH-926 and BT-474

Profiling of p53-responsive genes has traditionally been carried out in cellular models which require genetic modification or cytotoxic stimulation for p53 activation.[8–15,21–24] These approaches may be hampered by the possibility of biases related to viral infection, clonal isolation, non-physiologic overexpression and off-target effects. We sought to test whether IPH-926 and BT-474 cells allow for profiling of p53-responsive genes without genetic or cytotoxic manipulation. In particular, we were interested in defining

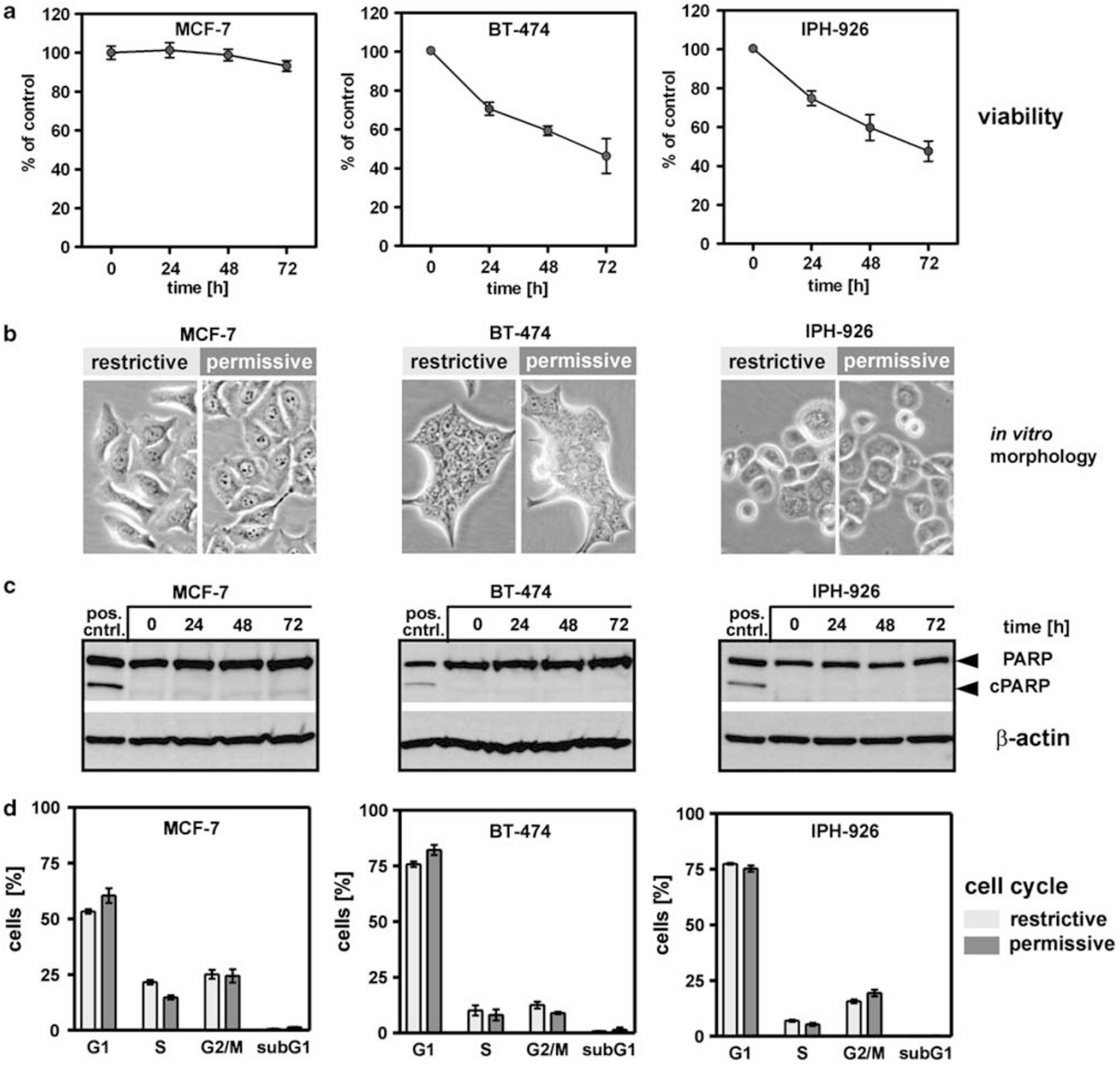

Figure 4 Reconstitution of endogenous wt p53 activity fails to induce apoptosis in IPH-926 and BT-474. (**a**) Cells were cultured at restrictive (37.5 °C) and permissive condition (32.5 °C) for different periods of time and viability was determined with the WST-1 assay. Symbols represent relative viability at permissive condition expressed as percent of control cells cultured at restrictive condition. Error bars represent s.e.m. (**b**) Unaltered *in vitro* morphology of breast cancer cells cultured at permissive condition (magnification, ×400). (**c**) Absence of PARP-cleavage, indicative of apoptosis. Cells were cultured at permissive condition for different periods of time. Total cellular protein was separated by 12% SDS–PAGE and was probed with a monoclonal anti-PARP antibody. Detection of β-actin verified equal loading. Positive controls (pos. cntrl.) for cleaved PARP (cPARP) were loaded on the first lane. (**d**) Absence of sub-G1 populations. Cells were cultured at restrictive and permissive condition for 24 h and were subjected to cell-cycle analysis. Bars represent the mean size of G1, S, G2/M and sub-G1 populations. Error bars represent s.e.m. Similar results were obtained after longer incubation periods at permissive condition (data not shown).

common transcriptional alterations associated with restored endogenous, basal wt p53 activity in the two breast cancer cell lines harboring ts-p53 E285K. Accordingly, three batches of IPH-926 cells were seeded into two parallel culture dishes each and were allowed to adapt to restrictive and permissive condition for 24 h before analysis on Affymetrix microarrays (Figure 5a). To gate out non-specific temperature effects, the same experiment was also performed with MCF-7 cells. Probe sets differentially expressed at restrictive *vs* permissive condition in MCF-7 were considered as non-specifically regulated. These probe sets were all filtered out of the IPH-926 expression data (Figure 5a). Using the filtered IPH-926 data and a $P = 0.0005$ significance threshold, two-class comparison analysis identified 68 probe sets corresponding to 60 genes as

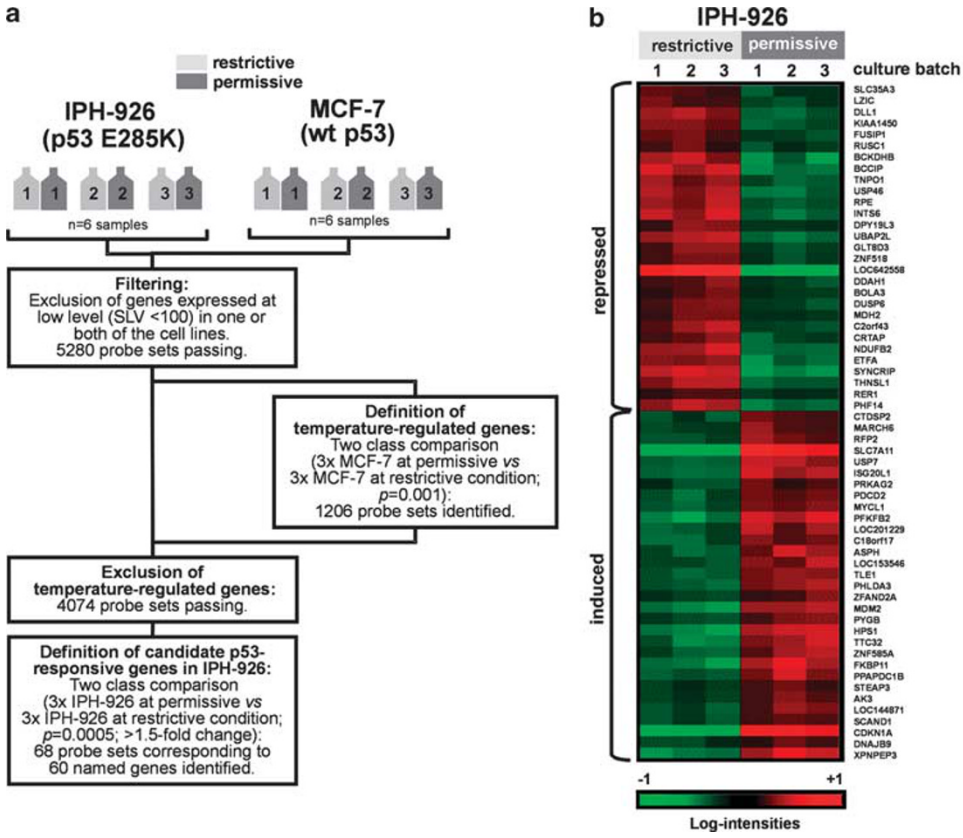

Figure 5 Microarray profiling of transcriptional alterations associated with restored endogenous wt p53 activity. (**a**) Experimental strategy. Three culture batches (1–3) of IPH-926 were incubated in two parallel culture dishes at restrictive and permissive condition for 24 h and were subjected to transcriptional profiling using Affymetrix microarrays. The same experiment was performed with MCF-7. Subsequent statistical analysis of IPH-926 expression data was carried out as described in the flow chart, using MCF-7 expression data to define and exclude probe sets, which were non-specifically regulated by temperature. (**b**) Two-dimensional presentation of gene expression pattern of 60 non-redundant genes identified as differentially expressed in IPH-926 at restrictive *vs* permissive condition. Each column represents an individual cell culture and each row represents a probe set. Gene symbols are aligned on the right side. Expression levels above the mean of individual probe sets are shown in red and expression levels below the mean are shown in green. A color scale corresponding to fold change is shown at the bottom. The same experiment was also performed with BT-474 and MCF-7 (see Supplementary Table 2).

differentially expressed in IPH-926 at restrictive *vs* permissive condition (Figure 5b; Supplementary Table 2). This experiment was also carried out with BT-474 and MCF-7 cells, resulting in the identification of 257 probe sets corresponding to 208 genes (Supplementary Table 3). A survey of p53 target gene inventories deduced from various cellular models revealed that the genes identified by this approach included several previously described p53-responsive genes, such as *CDKN1A, MDM2, SULF2, DDB2, PHLDA3*, *ISG20L1*, *RPS27L*, *C12orf5/TIGAR*, *TNFRSF10B/TRAIL-R2, STEAP3*, *KIF4A* and *CCNA2* (Figure 6a). A total of 15 genes were concordantly regulated at permissive condition in IPH-926 and BT-474. From these transcripts, we selected three known p53-responsive genes (*PHLDA3*, *ISG20L1*, *MDM2*) and three genes previously not associated with p53 (*TTC32, ASPH, PHF14*) for validation by quantitative real-time RT–PCR. In MCF-7 cells, cultivation at permissive condition did not influence expression of any of the genes tested (Figure 6b). In IPH-926 and BT-474 cells harboring ts-p53 E285K, cultivation at permissive condition induced an upregulation of *PHLDA3, ISG20L1, TTC32* and *MDM2*, while *PHF14* was moderately repressed (Figure 6b). Hence, IPH-926 and BT-474 facilitated microarray-based monitoring of p53-responsive genes without genetic or cytotoxic manipulation. Reconstitution of endogenous wt p53 activity was associated with partially similar transcriptional alterations involving known p53-responsive genes and transcripts not associated with p53 function, so far.

Similar Transcriptional Alterations in ASPC1 p53-Null Cells Transduced with ts-p53 A138V

Next, another cellular model was employed for studying p53-dependent regulation of genes identified in IPH-926 and BT-474. ASPC1 p53-null pancreatic cancer cells retrovirally transduced with ts-p53 A138V (ASPC1-ts-p53A138V-subclones A and B) are an established model for studying p53 function and were chosen for further analyses.[40] Cultivation at permissive condition upregulated $p21^{Waf1}$ in ASPC1-ts-p53A138V cells, which confirmed proper function of this

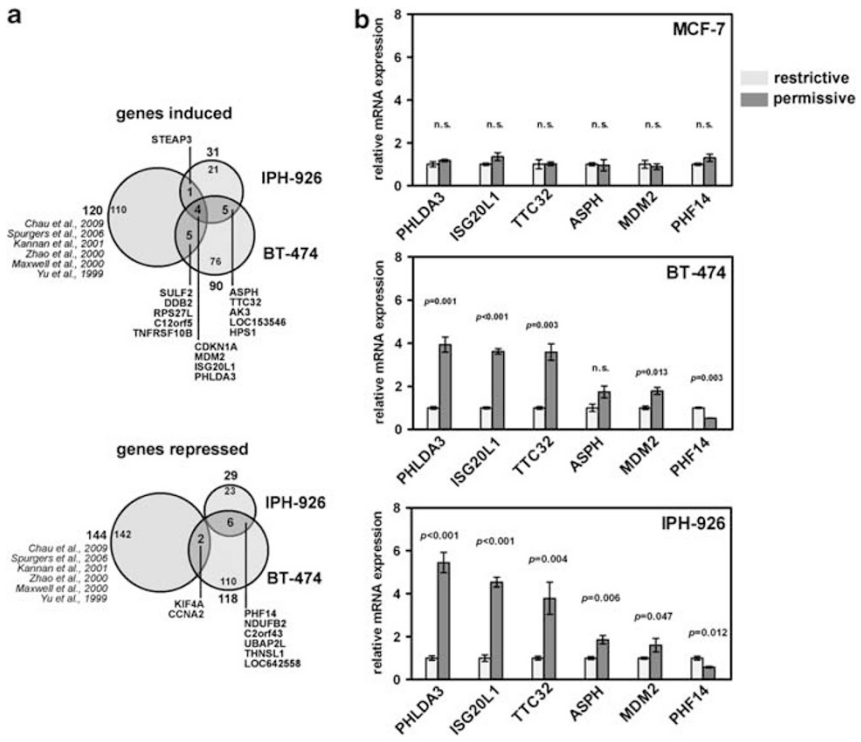

Figure 6 Meta-analysis and validation of genes identified by microarray profiling. (**a**) Genes differentially expressed in IPH-926 and BT-474 cells cultured at restrictive (37.5 °C) *vs* permissive condition (32.5 °C) were matched with compiled p53 target gene inventories deduced from six different models.[10,11,14,15,21] Numbers correspond to non-redundant genes. Symbols are given for genes overlapping between IPH-926, BT-474 or the reference inventory. (**b**) Cells were cultured for 24 h at restrictive and permissive condition and mRNA expression of the genes indicated was determined by quantitative real-time RT–PCR. Data are presented as mean relative expression with mRNA ratios of cells cultured at restrictive condition adjusted to a value of 1. Error bars represent s.e.m., n.s.: not significant.

model (Figure 7a). In parental ASPC1 cells transduced with an empty vector, permissive condition failed to influence expression of any of the genes tested (Figure 7b). In contrast, four of six genes tested were upregulated at permissive condition in ASPC1-ts-p53A138V cells, corroborating that those transcripts were modulated by p53 (Figure 7c). Like in IPH-926 and BT-474, p53-responsive genes included *TTC32*, which has not been associated with p53, so far (Figure 7c). However, p53-dependent upregulation of *MDM2* was much more pronounced in the ASPC1 cell model (Figures 6b and 7c). Upregulation of *PHLDA3* and *ISG20L1* was similar in IPH-926, BT-474 and ASPC1-ts-p53A138V cells (Figures 6b and 7c). Thus, IPH-926, BT-474 and ASPC1-ts-p53A138V cells showed partially similar characteristics with respect to gene expression changes upon reconstitution or ectopic overexpression of p53.

Low *PHLDA3* Expression is Associated with an Aberrant p53 Status in Primary Breast Cancers

Finally, p53-responsive genes were examined in primary breast cancers. To this end, we focused on *PHLDA3*. *PHLDA3* is a recently described inhibitor of AKT and demonstrates anti-tumor activity.[54] This gene was chosen for three reasons: (i) *PHLDA3* mRNA showed a pronounced upregulation to a stable plateau upon reconstitution of endogenous, basal wt p53 activity in IPH-926 and BT-474 (Supplementary Figure 1c). This implies that loss of wt *TP53* diminishes *PHLDA3* expression, irrespective of abrogating *PHLDA3* inducibility under external stresses, such as exposure to DNA-damaging agents. (ii) Kawase *et al*[54] have shown an upregulation of *PHLDA3* in MDA-MB-468 breast cancer cells with ectopic overexpression of wt p53. Together with our findings, this supports that p53-dependent regulation of *PHLDA3* is physiologically relevant in breast cancer. (iii) *PHLDA3* expression has not been examined in primary breast cancers before. Hence, a small collection of $n = 46$ mammary carcinomas was composed of selected cases showing either a strong, uniform nuclear p53 accumulation (indicative of a deleterious *TP53* mutation) or a weak, scattered p53 immunoreactivity, as typically seen in tumors harboring wt *TP53* (Figure 8a). None of the tumors had received neo-adjuvant anti-cancer therapy. *PHLDA3* mRNA expression was determined for all cases (Figure 8b). In this small collection of tumors, low or absent *PHLDA3* expression was clearly associated with nuclear p53 accumulation, suggesting that basal wt p53 activity is relevant for maintaining *PHLDA3* expression in human primary breast cancers (Figure 8c).

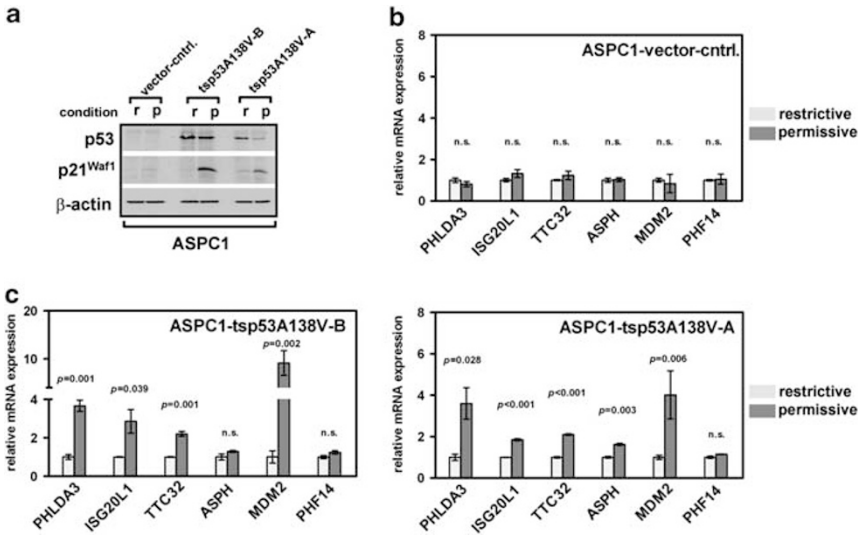

Figure 7 Similar transcriptional alteration in ASPC1 p53-null pancreatic cancer cells transduced with ts-p53 A138V. (**a**) Parental ASPC1 cells transduced with an empty vector (vector-cntrl.) and two ASPC1 subclones transduced with ts-p53 A138V (named ts-p53A138V-A and -B) were cultured for 24 h at restrictive (37.5 °C) and permissive condition (32.5 °C). Total cellular protein was separated by 12% SDS–PAGE and was probed with anti-p53 and anti-p21^{Waf1} antibodies. Detection of β-actin verified equal loading. (**b**, **c**) Cells were cultured for 24 h at restrictive and permissive condition and mRNA expression of the genes indicated was determined by quantitative real-time RT–PCR. Data are presented as mean relative expression with mRNA ratios of cells cultured at restrictive condition adjusted to a value of 1. Error bars represent s.e.m., n.s.: not significant.

DISCUSSION

Profiling of p53-responsive genes has traditionally been carried out in cellular models which require genetic modification or cytotoxic stimulation for p53 activation. The present study establishes an alternative approach, which circumvents genetic modification or cytotoxic stimulation. This approach is based on IPH-926 and BT-474 breast cancer cells. Both cell lines harbor a homozygous *TP53* missense mutation encoding for the rare p53 mutant E285K. For BT-474, this mutation had been described before, but has never been employed for the purpose of gene expression profiling.[50,51] For IPH-926 cells, which were established in our laboratory,[28,29] this is the first report describing an endogenous ts-p53 E285K mutation. Owing to analysis of recombinant proteins, p53 E285K has long been known to possess temperature-sensitive loss of function characteristics.[26,27] However, human cancer cell lines harboring this ts-p53 mutant intrinsically have rarely been used for studying p53 function. So far, only Mueller and Vojtesek and colleagues have utilized BT-474 to characterize p53 in mutant *vs* wt conformation.[50] They demonstrated that Hsp90 blocks MDM2-mediated degradation of mutant p53.[50] Here, we showed that cultivation of IPH-926 and BT-474 cells at permissive condition reconstitutes their endogenous wt p53 activity, as evidenced by induction of *CDKN1A* mRNA and p21^{Waf1}. In MCF-7 breast cancer cells harboring wt p53, cultivation at permissive condition had no such effect. The kinetics of *CDKN1A* mRNA upregulation in IPH-926 and BT-474 showed that a new equilibrium of endogenous wt p53 activity had been reached $\sim$24 h after shifting the cells to permissive condition. Interestingly, reconstitution of endogenous wt p53 activity did not induce apoptotic cell death in IPH-926 and BT-474. This is consistent with previous findings, indicating that p53 induces apoptosis only when permanently expressed at levels much higher than the endogenous, basal expression.[55] Subsequently, IPH-926 and BT-474 cells were employed to test the feasibility of a new approach for microarray-based assessment of p53-responsive genes. Two aspects were new about this experimental strategy. First, we utilized human breast cancer cells harboring endogenous ts-p53 cultivated at restrictive and permissive condition. This eliminated the need for genetic modification or cytotoxic stimulation to recover p53 activity and thus also excluded potential biases inherent to these manipulations. Second, we used an independent control cell line harboring wt p53 to account for non-specific temperature effects associated with the two cultivation conditions. Final statistical analyses of the microarray expression data were restricted to only those probe sets, which were not influenced by culture conditions in the control cell line. This approach readily identified upregulation of known p53 target genes like *CDKN1A* and *MDM2* in IPH-926 and BT-474 following reconstitution of endogenous, basal wt p53 activity. Quantitative real-time RT–PCR analyses confirmed similar transcriptional alterations in p53-null ASPC1 cells retrovirally transduced with ts-p53 A138V. Hence, IPH-926 and BT-474 cells indeed provided a useful new tool to study transcriptional programs controlled by p53. It is likely that the full lists of transcripts identified by our profiling approach include numerous previously unrecognized p53-responsive genes, such as *TTC32*. IPH-926 and BT-474 provide an attractive model for future studies in this direction.

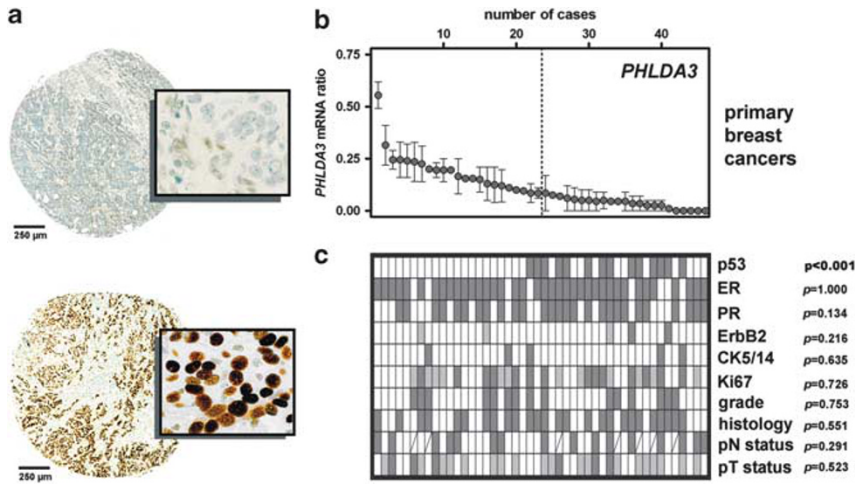

Figure 8 Low or absent *PHLDA3* expression is associated with nuclear p53 accumulation in human primary breast cancers. (**a**) Representative immunohistochemical stainings of a mammary carcinomas with nuclear p53 accumulation or weak, scattered p53 reactivity, as carried out on tumor tissue microarrays. (**b**) *PHLDA3* mRNA expression was determined by quantitative real-time RT–PCR using two housekeeping genes (*β-GUS* and *TBP*) for normalization as decribed in the Materials and methods section. Each dot in the diagram represents a primary mammary carcinoma. Cases are ranked by *PHLDA3* mRNA ratio. Error bars represent s.e.m. The dotted line indicates the sample median dividing the tumor cohort in groups with high/moderate or low/absent *PHLDA3* expression. (**c**) Two-dimensional presentation of clinicopathological characteristics or primary breast cancer specimens. Sample order is the same as in the diagram above. Dark gray indicates: p53; nuclear accumulation (cutoff $\geq$90%, intensity scored 3), ER; positive, PR; positive, ErbB2; 3+, CK5/14; positive, Ki67; LI $\geq$25%, grade; G3, histology; ductal/NOS, pN status; 1+, pT status; pT3/4. Light gray indicates: Ki67; $<$25 and $\geq$10%, ErbB2; 2+ (all ErbB2 FISH-negative), pT status; pT2. White indicates: p53; weak and scattered immunoreactivity, ER; negative, PR; negative, ErbB2; 0/1+, CK5/14; negative, Ki67; LI $<$10%, grade; G1/2, histology; lobular, pN status; pN0, pT status; pT1. Cases with a pNx status are indicated by a diagonal line. Statistical significance of the association of clinicopathological characteristics with low/absent *PHLDA3* expression was determined by the χ^2 test and the χ^2 test for trends for ErbB2, Ki67 LI, and the pT status. Different cutoffs for p53 nuclear accumulation, such as 20 or 40%, yielded similar results in this tumor collection (not shown). Please note that the 6th case from the left side corresponds to a triple-negative, G3, pleomorphic lobular carcinoma, which, however, did not show a nuclear accumulation of p53.

One overt advantage of this model is that p53-dependent gene regulation can be studied dynamically in the states of p53 inactivation, p53 hyper-activation, and endogenous basal p53 activation. This is impossible in other models, such as ASPC1 cells transduced with ts-p53 A138V, because p53 expression is fixed at high levels by the ectopic expression construct and the endogenous basal p53 activation level is elusive. One potential disadvantage of our model for studying p53-responsive genes is the need for an external control cell line, which may slightly influence the population of genes that pass the initial filtering process. Also, we cannot exclude that some genes regulated by both, temperature and p53, were excluded from further analysis. Nonetheless, the possibility to culture IPH-926 and BT-474 cells in disparate states may also support other experimental applications for target gene identification like chromatin immunoprecipitation. However, to this end, we focused on *PHLDA3*. This gene encodes an inhibitor of AKT and was upregulated at permissive condition in IPH-926, BT-474 and ASPC1 cells transduced with ts-p53 A138V. Kawase *et al*[54] have also shown an upregulation of *PHLDA3* in MDA-MB-468 cells transduced with wt p53 and characterized a p53-binding motif in the *PHLDA3* promoter region. Together with our findings, these results suggest that p53-dependent regulation of *PHLDA3* is physiologically relevant in breast cancer. To further investigate this, *PHLDA3* mRNA expression was examined in a small collection of human primary breast tumors for the first time. Low or absent *PHLDA3* expression was associated with nuclear p53 accumulation, indicative of deleterious *TP53* mutations. In line with our *in vitro* model based on IPH-926 and BT-474, this indicates that basal wt p53 activity is relevant for maintaining *PHLDA3* expression in human primary breast cancer. However, this work establishes more than just a mechanistic model for p53 research. Of note, IPH-926 cells originate from an ILC.[28] These rather indolent tumors develop through a so-called low-grade molecular progression pathway and usually lack *TP53* alterations.[4,56] Other kinds of breast cancers, such as aggressive basal-like mammary carcinomas, develop through a separate pathway, termed high-grade pathway, and harbor *TP53* mutations.[2,56] These two progression pathways have been thought to be mutually exclusive.[36,56] However, it has been problematic to assign pleomorphic infiltrating ILCs (PLCs), an aggressive variant of classical ILCs, to either the low-grade or the high-grade progression pathway.[36] Remarkably, these tumors may show characteristics of classical ILCs, including inactivation of *CDH1* encoding for E-cadherin, combined with characteristics of high-grade mammary carcinomas, including mutation of *TP53*.[33,35,36,39] It has been speculated that PLCs arise from classical low-grade lobular mammary carcinomas as they switch to the high-grade progression pathway and that inactivation of *TP53* could drive this process.[36,38,56] Except

for a convincing mouse model, this has been hypothetical, so far.[36,38,56] The findings presented herein demonstrate that the primary ILC corresponding to IPH-926 had undergone such a secondary transition. As shown in Table 1, the ts-p53 E285K mutation present in IPH-926 had evolved as a late, secondary genetic event during the *in vivo* clonal evolution of a classical lobular mammary carcinoma with a unique *CDH1* frameshift mutation but a wt *TP53* sequence. Moreover, our *in vitro* data highlight that one consequence of this secondary p53 abrogation had been a downregulation of *PHLDA3*. From a tumor pathology perspective, IPH-926 ILC cells thus provide the first direct evidence for the real existence of a p53-related clonal evolutionary pathway in ILC progression, which may involve a dysregulation of *PHLDA3*. Yet, this does not mean that all ILCs progress exactly like this. Three other, similar cases of primary classical ILCs matched with metachronous recurrences encountered at our institution did not show a secondary transition to PLC at the time of recurrence. Also, a single ILC matched with its metachronous metastasis, which has recently been the subject of a widely recognized publication in the *Nature* science journal, did not show a secondary transition to PLC.[57] The fact that still about 50% PLCs retain wt *TP53* suggests that inactivation of other tumor suppressor genes or activation of additional oncogenes may have cell biological effects similar to those induced by abrogation of p53.[39] Theoretically, these alternative alterations could likewise drive ILC progression or a secondary transition to PLC. With these restrictions in mind, one can say that the findings reported herein are important for two reasons: First, because they further clarify the genetic and functional properties of a cell model, which will be relevant for future research. Second, because they prove the real existence of a distinct molecular evolutionary pathway in ILC, which has been hypothetical, so far.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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