Expression of the BRAFV600E oncoprotein is known to cause benign lesions, such as melanocytic nevi (moles). Despite the oncogenic function of mutant BRAF, these lesions are arrested by a cell-autonomous mechanism called oncogene-induced senescence. Infrequently, nevi can progress to malignant melanoma, through mechanisms that are incompletely understood. To gain more insight into this vital tumor-suppression mechanism, we performed a mass-spectrometry-based screening of the proteome and phosphoproteome in cycling and senescent cells and in cells with abrogated senescence. Proteome analysis of senescent cells revealed the up-regulation of established senescence biomarkers, including specific cytokines, but also several proteins not previously associated with senescence, including extracellular matrix-interacting. Using both general and targeted phosphopeptide enrichment by Ti4⁺ IMAC and phosphorylase antibody enrichment, we identified over 15,000 phosphorylation sites. Among the regulated phosphorylation sites we encountered components of the interleukin, BRAF/MAPK, and CDK-retinoblastoma pathways and several other factors. The extensive proteome and phosphoproteome dataset of BRAFV600E-expressing senescent cells provides molecular clues as to how oncogene-induced senescence is initiated, maintained, or evaded, serving as a comprehensive proteomic basis for functional validation. 

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In order to sustain their reproductive life spans, multicellular organisms require several safeguard mechanisms to maintain cell homeostasis. Growth and cell replication are essential processes; however, uncontrolled growth can be detrimental, resulting in cancer and eventually death. Therefore, several tumor-suppressive mechanisms have evolved, such as apoptosis (1) and replicative senescence (2, 3), which can lead to cell self-destruction and cell proliferation arrest, respectively. Recently it has been shown both in vitro (4–6) and in vivo (7–9) that oncogene activation (e.g. through BRAF, Ras, Raf, and E2F) can induce an irreversible cell-growth-inhibition mechanism termed oncogene-induced senescence (OIS).¹

Human skin cells are naturally exposed to multiple stress factors that can induce gene mutations, which can potentially lead to constitutive protein activation and ultimately tumor formation. Benign skin tumors that may remain dormant for decades are manifested throughout the human skin in the form of melanocytic nevi (moles) and rarely progress into a malignant state. Nevi show markers of senescence including growth arrest, increase in senescence-associated (SA)-β-galactosidase activity, and induction of tumor suppressor p16 (7). Strikingly, the BRAFV600E activating mutation (10) is found with very high frequency (~50%) in both nevi and primary melanomas (11). This suggests that this BRAF mutation alone is insufficient for melanoma development and additional mutations or other post-transcriptional alterations are needed for transformation. This idea is supported by BRAFV600E knock-in mouse models, which develop noncongenital nevi that frequently progress to melanomas (12, 13). Transcriptomic analysis has previously shown that the maintenance of BRAFV600E-induced senescence is dependent on an inflammatory network governed by the transcription factor CCAAT-enhancer-binding protein β (C/EBPβ) (14). Senescence mechanisms defying tumor outgrowth are currently being heavily

¹ The abbreviations used are: OIS, oncogene-induced senescence; ECM, extracellular matrix; HDF, human diploid fibroblast; IMAC, immobilized metal affinity chromatography; OISb, oncogene-induced senescence bypass; SCX, strong cation exchange; SA, senescence-associated; C/EBPβ, CCAAT-enhancer-binding protein β; FA, formic acid; ACN, acetonitrile; STAT3, signal transducer and transcription activator 3.
investigated with regard to the endogenous tumor-suppressor pathways involved and alternative drug solutions to cancer treatment.

To better understand at the molecular level the mechanisms underlying the onset and maintenance of OIS in human fibroblasts, we used multiple complementary proteomics techniques to achieve a high coverage of both the proteome and the phosphoproteome (15). Each protocol has been optimized previously (16–18) to maximize the number of proteins and phosphorylation events quantified. Strong cation exchange peptide fractionation together with both global phosphopeptide enrichment and phosphotyrosine site-specific enrichment techniques was applied to allow for a deep coverage of the senescence phosphoproteome. Using stable isotope dimethyl labeling, we were able to quantify 5,997 proteins, 12,547 phosphoserine sites, 2,361 phosphothreonine sites, and 590 phosphotyrosine sites.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Assays**—The human diploid fibroblast (HDF) cell line Tg3 expressing the ectopic receptor hTERT and sh-p16INK4A (Tg3 (et)-16i) was maintained in DMEM with 4.5 mg/ml glucose and 0.11 mg/ml sodium pyruvate supplemented with 9% fetal bovine serum (PAA, Pasching, Austria), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). The Phoenix packaging cell line was used for the generation of ecotropic retroviruses. The plasmids pMSCV-bl and pMSCV-bl-BRAFV600E, as well as pRS-puro and pRS-puro-C/EBPδ, were previously described (14).

A representative results of several independent experiments.

**Phosphoproteomics Sample Preparation**—Frozen cell pellets were lysed by sonication in lysis buffer (8 M urea in 50 mM ammonium bicarbonate, 1 tablet Complete Mini EDTA-free Mixture (Roche), and 1 tablet PhosSTOP phosphatase inhibitor mixture (Roche)); samples for phosphotyrosine peptide pulldowns contained additional 1 mM sodium orthovanadate. After centrifugation (20,000 g for 30 min at 4 °C), the supernatant was assayed for protein content using the BCA kit (Pierce). Protein reduction and alkylation were performed using final concentrations of 5 mM dithiothreitol and 10 mM iodoacetamide, respectively. A first enzymatic digestion step was performed at days 3 and 9 after the last infection. Images of cell proliferation assays were performed in 8 M urea lysis buffer using Lys-C at 37 °C for 4 h (enzyme:substrate = 1:75). The second digestion was performed overnight (37 °C) with trypsin (enzyme:substrate = 1:100) in 2 M urea. Resulting peptides were chemically labeled and washed on Sep-Pak C18 columns (Waters, Milford, MA) using stable isotope dimethyl labeling as described previously (19). Cycling HDF cells were labeled with light, OIS cells with medium, and OIS bypass cells with heavy dimethyl isotopes. In the replicate experiment, the medium and heavy labels were swapped. The labeling efficiency for all labels was greater than 95%. An aliquot of each label was measured on a regular LC-MS/MS run, and samples were mixed 1:1:1 (L:Hi:H) based on their peptide intensities. This was found to result in a more precise ratio than using the total protein amounts as determined by a BCA assay. After mixing, peptides were dried to completion in vacuo.

**PolyCUD Phosphopeptide Enrichment**—Peptides were suspended in 800 µl of cold immunoaffinity purification buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, protease inhibitors (Roche Diagnostics, Germany), and 1% n-octyl-β-D-glucopyranoside (Sigma). The peptide mixture was agitated on a shaker for 30 min to dissolve the peptides thoroughly, and the pH was adjusted, if necessary, to pH 7.4. The immunoaffinity purification was performed as previously described (20, 21). In summary, the peptides were added to 50 µl of a slurry of PY99 antibody beads (Santa Cruz Biotechnology, Santa Cruz, CA), and the peptide-antibody bead mixture was incubated overnight at 4 °C on a rotator. The beads were spun down, and the supernatant was used for a second iteration of immunoaffinity purification using fresh PY99 beads. The pelleted beads were washed, and bound peptides were eluted twice using 0.15% trifluoroacetic acid (TFA) (Sigma). Sample desalting was performed using homemade tips with C18 material (Aquata™ C18, 5 µm, Phenomenex, Torrance, CA) as described elsewhere (22). Finally, peptides were dried in vacuo and reconstituted in 40 µl of 10% formic acid prior to LC-MS/MS analysis.

**Strong Cation Exchange Chromatography for Phosphate Fractionation**—SCX was performed on two separate systems optimized for sample amount and type. For the protein identification experiment, 150 to 200 µg of sample was injected on SCX system 1, and for the Ti⁺-IMAC phosphopeptide identification experiment, 3 mg was loaded on SCX system 2. For SCX system 1, peptides were fractionated as described elsewhere (65).Briefly, the SCX system consisted of an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with a Strata X 33u (Phenomenex, Utrecht, The Netherlands; 50 × 4.6 mm) trapping cartridge and a polysulfoethyl A SCX column (PolyLC, Columbia, MD; 200 mm × 2.1 mm, 5 µm, 200 Å). Labeled peptides were reconstituted in 10% formic acid (FA), loaded onto the trap column at 100 µl/min, and subsequently eluted onto the SCX column with 80% acetonitrile (ACN) (Biosolve, Valkenswaard, The Netherlands) and 0.05% FA. SCX buffer A was made out of 50 mM KH₂PO₄ (Merck, Darmstadt, Germany), 50% ACN, and 0.05% FA, pH 2.7; SCX buffer B consisted of 350 mM KCl (Merck), 5 mM KH₂PO₄, 30% ACN, and 0.05% FA, pH 2.7. The gradient was as follows: 0% B for 10 min, 0% to 85% B in 35 min, 85% to 100% B in 6 min, and 100% B for 4 min. A total of 45 fractions were collected for each set and dried in a vacuum centrifuge. The second SCX system (66) used an Opti-Lynx (Optimized Technologies, Oregon City, OR) trapping cartridge and a Zorbax BioSCX-Series II column (0.8-mm inner diameter × 50-mm length, 3.5 µm). SCX solvent A consisted of 0.05% FA in 20% ACN, and solvent B was 0.05% FA, 0.5% NaCl in 20% ACN. The SCX salt gradient was as follows: 0–0.01 min (0% to 2% B); 0.01–8.01 min (2% to 3% B); 8.01–14.01 min (3% to 8% B); 14.01–28 min (8% to 20% B); 28–38 min (20% to 40% B); 38–48 min (40% to 90% B); 48–54 min (90% B); 54–60 min (0% B). A total of 50 SCX fractions (1 min each, 50-µl elution volume) were collected and dried in a vacuum centrifuge.

**Ti⁺-IMAC Phosphopeptide Enrichment**—Prior to phosphopeptide enrichment, SCX fractions were desalted using Sep-Pak C18 columns and dried to completion using a speed vac. Ti⁺-IMAC columns were prepared and used as described previously (18, 23). Briefly, microcolumns were created by loading Ti⁺-IMAC beads onto GEloader tips (Eppendorf, Hamburg, Germany) with a C8 plug to −1- to 2-cm length. The enrichment procedure for all SCX fractions was as follows: Ti⁺-IMAC columns were pre-equilibrated two times with 2090
30 μl of loading buffer (80% ACN, 6% TFA). Next, each SCX fraction was resuspended in 30 μl of loading buffer and loaded onto the equilibrated GELoader tips. TiF⁺-IMAC columns were washed with 40 μl of washing buffer A (50% ACN, 0.5% TFA, 200 mM NaCl) and subsequently with 40 μl of washing buffer B (50% ACN, 0.1% TFA). Bound peptides were eluted by 30 μl of 10% ammonia into 30 μl of 10% FA. Finally, the remainder of the peptides was eluted with 4 μl of 80% ACN, 2% FA. The collected eluate was further acidified by 6 μl of 100% FA and subsequently stored at −20 °C for LC-MS/MS analysis.

Liquid Chromatography and Mass Spectrometry—For protein identification and quantification, the SCX fractions containing doubly and triply charged peptides (approximately 20 fractions for each SCX) were reconstituted in 10% FA and analyzed directly via nanoflow reverse phase liquid chromatography using an Agilent 1200 coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo, San Jose, CA). Depending on the SCX UV trace, 1% to 10% of each fraction was injected. Densely populated 2+ fractions were injected twice to minimize undersampling of the mass spectrometer. About half of each phosphopeptide sample was injected to allow for erroneous events. Peptides were trapped on a trap column (ReproSil-Pur C18-AQ, 3 μm, Dr. Maisch GmbH, Ammerbuch, Germany; 20 mm × 100 μm inner diameter, packed in-house) at 5 μl/min in 100% solvent A (0.1 M acetic acid in water). Next, peptides were eluted from the trap column onto the analytical column (ReproSil-Pur C18-AQ, 3 μm, Dr. Maisch GmbH; 40 cm × 50 μm inner diameter, packed-in-house) at ~100 nl/min in 1-h, 2-h, or 3-h linear gradients from 10% to 50% solvent B (0.1 M acetic acid in 8:2 (v/v) acetonitrile:water). Nanospray was achieved with an in-house pulled and gold-coated fused silica capillary (360 μm outer diameter, 20 μm inner diameter, 10 μm tip inner diameter) and an applied voltage of 1.7 kV. In all experiments the mass spectrometer was configured to perform a Fourier transform survey scan from 350 to 1500 m/z at a resolution of 30,000. For the protein identification/quantification experiment, the top 10 most intense peaks were fragmented via higher energy collision-induced dissociation fragmentation (35% normalized collision energy at a collision energy bypass (OISb) cells were harvested 9 days after BRAFV600E infection. Cells were scraped with 1× PBS and centrifuged at 4000 rpm for 4 min at 4 °C, and the pellets were frozen or used immediately. Fresh or frozen pellets were lysed on ice in RIPA buffer supplemented with protease inhibitor mixture (Roche) and phosphatase inhibitors (10 mM β-glycerophosphate, 2 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM sodium pyrophosphate). Lysates were sonicated for 1 min (5-s on/off interval) and centrifuged at 4 °C and 1200 rpm for 10 min, and supernatants were transferred to fresh Eppendorf tubes. Protein concentrations were determined via Bradford assay (Bio-Rad). Protein samples were prepared in 4× sample buffer (Invitrogen) supplemented with 2.5% β-mercaptoethanol. Proteins were separated on 4–12% polyacrylamide gels (Invitrogen), transferred onto a nitrocellulose membrane (Whatman), and blocked in blocking buffer (4% milk in 1× TBS-Tween) for 1 h at room temperature. The membrane was probed with the indicated primary antibodies (overnight at 4 °C in 4% milk in 1× TBS-Tween) and then incubated for 1 h with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) enzyme. For detection of the signal, the membrane was incubated for 1 min with ECL reagent (Amersham Biosciences) and visualized on films (GE Healthcare).

Antibodies—The primary antibodies used for immunoblot analysis were C2CD2 (D01P; H00025966; MaxPab, Abnova, Taipei City, Taiwan), phospho-cdc2 (known as CDK1) (Tyr15) (#9115; Cell Signaling), cdc2 (known as CDK1) (#9112; Cell Signaling), FGF-2 (C-18; sc-13604; Santa Cruz), Hsp90 (4874; Cell Signaling), STAT-3 phospho (Tyr705) (SE2; #9138; Cell Signaling), and STAT-3 (sc-482; Santa Cruz). Secondary antibodies used were goat anti-mouse IgG (H+L) HRP conjugated (G21204; Invitrogen), goat anti-rabbit IgG (H+L) HRP conjugated (G21324; Invitrogen), and rabbit anti-goat IgG (H+L) HRP conjugated (R-21459; Invitrogen).

RESULTS

Model System—To study the mechanisms underlying OIS, we employed a previously described cell system (14) comprising in vitro cultured HDFs in three differential states: cycling, OIS, and OISb. HDFs transduced with lentivirus harboring an empty vector were used as normal cycling control cells. OIS was induced by infecting HDFs with a lentivirus carrying
the constitutively active \(BRAF^{V600E}\) gene. OIS was abrogated in the presence of mutated BRAF by depletion of C/EBP\(\beta\), a crucial component of the inflammatory pathway in OIS (14). HDF cells were chosen as the model system because they are easily grown in the quantities required for proteomics experiments, and previous studies have shown that all observed factors crucial for OIS are validated in primary melanocytes or in vivo models (14, 29). Cells were additionally modified for ectopic hTERT expression and p16 silencing to prevent the stochastic induction of replicative senescence producing heterogeneous cell populations. Cells expressing the \(BRAF^{V600E}\) oncogene typically showed an initial burst of growth followed by the induction of senescence. Three days after infection, all cells showed equal proliferative kinetics (Fig. 1A). However, after 9 days, cells expressing \(BRAF^{V600E}\) displayed a strong growth arrest accompanied by the induction of the senescence marker SA-\(\beta\)-galactosidase, whereas the cycling control cells continued proliferation without displaying this senescence marker (Fig. 1B, Cycling and \(BRAF^{V600E}/\)Vector). As expected, the C/EBP\(\beta\)-depleted OISb cells, in contrast to OIS cells but similarly to cycling cells, failed to undergo cell cycle arrest or induce senescence markers by 9 days following \(BRAF^{V600E}\) introduction (Figs. 1A and 1B, \(BRAF^{V600E}/\)sh-C/EBP\(\beta\)). Therefore, we hypothesized that the comparison of cycling, OIS, and OISb samples in the early onset and end-point phenotypes would allow us to find proteins and phosphosites regulated specifically in the onset and maintenance of senescence. Moreover, the comparison between OISb and OIS cells could potentially reveal processes involved in the bypass of senescence into tumor progression/malignancy.

**Approach**—To uncover protein expression specific for cycling, OIS, or OISb, related lysates were digested and the resulting peptides were desalted and labeled with different dimethyl isotopes. After mixing of the three labeled cell lysates, peptides were processed through three distinct pipelines. For full proteome analysis, peptides were fractionated by SCX, and for unbiased global phosphopeptide analysis an additional SCX was run, followed by Ti\(^{4+}\)-IMAC enrichment as described before (18). For the specific analysis of tyrosine phosphorylation dynamics, the protein digest was directly treated with phosphotyrosine antibody-coupled beads (Fig. 1C). The workflow was applied to cells harvested 3 days and 9 days post-transduction, and the replicate experiment was performed using a label swap.

The first experiment was focused on the analysis of unmodified peptides to assess protein identity and protein expression differences and resulted, following stringent filtering as described in “Experimental Procedures,” in the robust quantification of 5,997 proteins. The extensive fractionation before the highly selective Ti\(^{4+}\)-IMAC phosphopeptide enrichment together with the pTyr immunoprecipitation resulted in the quantification of 18,320 unique phosphopeptides (15,498 unique phosphosites) with a localization certainty of on average 90% (Fig. 1D). Because of the different types of data distributions for different sample comparisons, we used a threshold of 2-fold up- or down-regulated in both replicates to pinpoint differentially regulated proteins/phosphopeptides. The full lists of all quantified proteins and phosphopeptides can be found in supplemental Tables S1 and S2, and the raw
files have been made available as supplementary data. The ratios determined in our three parallel experiments were used to define two sets of biologically distinct changes. The first set of regulated events was so-called BRAF specific and was observed when comparing both OIS cells and OISb cells to cycling cells. The second, and most interesting, scenario was termed OIS specific and consisted of features up- or down-regulated in comparisons of OIS versus cycling and OISb versus OIS. In order to find consistent regulations, we plotted the logarithmic base 2 ratio for both replicates in each sample comparison in Fig. 2.

Proteome Changes upon BRAF Expression—As displayed in the OIS versus OISb protein ratio plots, minor changes were observed after 3 days that became more apparent after 9 days when the full phenotypes were displayed (16 and 102 substantially regulated proteins after 3 and 9 days, respectively) (Fig. 2, Proteins; supplemental Fig. S1). Interestingly, larger numbers of differentially regulated proteins were observed for the OIS versus cycling and OISb versus cycling cells, and those numbers increased over time (93 and 387 proteins regulated at 3 and 9 days, respectively) (Fig. 2, Proteins; supplemental Fig. S1). Interestingly, larger numbers of differentially regulated proteins were observed for the OIS versus cycling and OISb versus cycling cells, and those numbers increased over time (93 and 387 proteins regulated at 3 and 9 days, respectively) (Fig. 2, Proteins; supplemental Fig. S1). Although the OIS cells were cell cycle arrested after 9 days and both the OISb and cycling cells were still growing, substantially greater similarity in protein expression was observed between OISb cells and OIS cells than between OISb and cycling cells. This illustrates that oncogene activation has clearly a dominant effect on global protein expression levels. In line with expectations, all BRAF-expressing cell lines displayed similarly elevated levels of BRAF protein expression after 3 and 9 days of growth, suggesting equal transduction levels (Fig. 2, Proteins OIS/OISb; supplemental Fig. S1).

Ontology enrichment analysis revealed that proteins concurrently down-regulated in all BRAF-expressing cells (OIS and OISb, 3 days and 9 days) are primarily involved in extracellular matrix interactions (Fig. 3A). Extracellular matrix constituents such as collagens (I, III, VI, XII), fibronectin, and cytoskeletal proteins filamin A and filamin C were among the most strongly down-regulated proteins in both OIS and OISb (Fig. 2, supplemental Table S1). Interestingly, there is a difference between the early and late stages of oncogenic insult for some down-regulated protein categories. DNA replication and cell cycle regulatory proteins were differentially enriched in BRAF-expressing cells at day 3, reflecting the early growth stimulatory response, whereas cell contact gene ontologies were specifically enriched in the late stage. The only significantly enriched protein group up-regulated in both OIS and OISb belonged to the lysosome compartment.

When looking more specifically into the data, it is interesting to note that IFI16 was up-regulated in all BRAF-expressing cells after 3 and 9 days (Fig. 2, supplemental Fig. S1). It has been shown previously that IFI16 is able to induce senescence via up-regulation of p21(CDKN1A) and that subsequent p21 knockdown can bypass senescence (30). In our model we saw p21 up-regulation in both OIS and OISb, but only after 9 days, suggesting a delayed tumor-suppressive response to...
oncogene activation. Moreover, in line with the up-regulation of p21, CDK1 protein expression levels showed down-regulation after 9 days in OIS that seemed to be less strong in OISb (Fig. 2, Fig. 4B, supplemental Fig. S1).

**Phosphoproteome Changes upon BRAF Expression**—Similar to the proteome changes, the phosphoproteome changes were found to be the largest when we compared either OIS or OISb cells to cycling cells (Fig. 2, supplemental Fig. S2). In line with an increase of BRAF protein expression, we also observed a strong increase in BRAF Ser729 phosphorylation in OIS and OISb cells. At the same time, phosphorylation on NF1 (S2543, T2565, S2802) and sprouty (related) proteins (Spry2 S167, Spry4 S125, Spred1 S238, and Spred2 S168) that are known to negatively affect BRAF activation were similarly up-regulated after BRAF oncogene introduction. This is in line with a previous study by Courtois-Cox et al. demonstrating a negative feedback signaling loop involved in OIS (31). In addition, up-regulation of ATM (T1885) phosphorylation was observed, although ATM protein levels remained unchanged (Fig. 2, supplemental Fig. S2). Although biological information about this specific site is absent, this site could be potentially interesting, as the ATM stress kinase is known to phosphorylate important components of the stress response such as p53 (32).

As described above, the increase in protein levels of the CDK inhibitor p21 was observed in both OIS and OISb only after 9 days. Therefore, we searched for the effects of p21 up-regulation on phosphorylation levels of CDK targets. Our global phosphorylation screen revealed a strong reduction in CDK-dependent phosphorylation sites, as observed in the sequence motif analysis (Fig. 4C). Relative to background, the CDK phosphorylation motif (SPxK) was strongly and solely enriched in the set of phosphosites down-regulated in both BRAF-expressing cells after 9 days (56-fold increase). When looking at individual CDK targets, RB1 is one of the major Cyclin/CDK substrate proteins controlling cell cycle progression; RB1 hypophosphorylation is associated with transcriptional repression, whereas hyperphosphorylation relieves its tumor-suppressive role. In line with this, strong down-regulation of RB1 phosphorylation sites in senescence was ob-
served, and the proteome screening indicated unchanged RB1 protein levels. After 3 days of growth, phosphorylated T356 and the dual phosphorylated sites S249, T252 and T821, T826 were down-regulated in the pre-OIS cells only (Fig. 2, supplemental Fig. S2). After 9 days, phosphorylation of both dual phosphorylation sites was nearly absent in both OIS and OISb but still present in cycling cells (supplemental Fig. S3). Furthermore, RB1 T356 was found to be down-regulated in OIS and, to a lesser extent, in OISb (Fig. 2B, supplemental Fig. S2). These changes in phosphorylation suggest that RB1 may play a partial role in the mutant BRAF-induced proliferative arrest.

Another CDK1 substrate is histone acetyltransferase Myst2 T88, which was strongly down-regulated in the OIS and OISb cells, whereas protein levels remained unregulated. Wu and Liu have shown that phosphorylation of this site by CDK1 can be crucial for cell cycle progression (33). Similarly, another CDK1 substrate, S38 on Stathmin1 (Stmn1) (34), showed down-regulation of phosphorylation in both OIS and OISb.

In summary, these findings indicate a strong tumor-suppressive response to mutant BRAF expression in this model system, involving CDK inhibition, leading to a potent cell cycle arrest. However, although all BRAF-expressing cells display greater sensitivity for cell cycle arrest, only the cells with intact C/EBPβ levels have the ability to enter a full cell cycle arrest. These senescence-specific events were studied next.

Proteome Changes Specific for Senescence—Even though the proteomes of (pre-)OIS and (pre-)OISb are very much alike at a global scale, the former cells are cell cycle arrested, whereas OISb cells keep proliferating. This implies there are additional molecular mechanisms that distinguish OIS cells from OISb cells. Therefore, we focused on a small set of proteins that also displayed differential expression between OIS and OISb in the early and full OIS stages (i.e. 3 and 9 days), thereby discriminating between markers of senescence onset and maintenance.

In Fig. 3 an overview is given of the senescence-specific proteins; in Fig. 3A they are clustered into gene ontology groups. Up-regulated enriched gene ontologies include mainly lysosomal, inflammatory, and extracellular acting proteins (Fig. 3A). To visualize the dynamics of senescence-associated proteins, we clustered the intensities for proteins regulated in OIS cells after 3 days and/or 9 days in Fig. 3B. Interestingly, some extracellular matrix processing proteins such as SerpinB2/PAI-2 and TFPI2 were regulated after both 3 and 9 days, whereas other proteins belonging to the same category were exclusively up-regulated after 3 days (SerpinB8) or 9 days (MMP1, TIMP1). This temporal regulation could indicate that different phases of extracellular modifications are required in order for the cell to progress into a senescence-like state.

Consistent with our previous results on mRNA profiling (14), strong up-regulation was found for the cytokines IL-1β and IL-8 in senescent cell lines at day 9. At the pre-OIS stage, IL-1β was already clearly visible in both BRAF-expressing cells, whereas IL-8 was identified in one replicate only. Additional proteins specifically regulated in the final senescent phenotype (Fig. 3C) included CEBP/B and TSC22D1, which
we have shown previously to stimulate the production of cytokines such as IL-1b and IL-8 and to be crucial in maintaining the senescence phenotype in OIS (14, 35, 36). Similarly, PTGS2 (COX2), the main enzyme in inflammation-linked prostaglandin production, was found to be up-regulated in OIS in our data and was shown previously to be critical for senescence onset and maintenance (37). Other up-regulated proteins included glucose-linked biomolecule-processing enzymes (i.e. AGA, GFPT2) and lysosomal (i.e. GAA, GLB1/β-galactosidase) proteins.

Another factor strongly up-regulated specifically in OIS was the fibroblast growth factor FGF2. The finding of this growth-promoting factor was at first sight counterintuitive, but Western blotting validated a strong presence of both the short excreted para-/autocrine FGF2 and the longer NLS-containing intracrine FGF2 variant of 18 kDa and 24 kDa, respectively (Fig. 4 C).

One of the most strongly down-regulated proteins in senescence was the deubiquitinating enzyme USP36 (Fig. 2, Fig. 3C). Interestingly, this enzyme was previously shown by Richardson et al. to positively control cell growth by stabilizing rRNA production and ribosome biogenesis (38). Moreover, knockdown of both yeast and fly USP36 homologs was shown to result in an inhibition of cell growth (38, 39). Although further biological validation is required, in light of our model system, USP36 can potentially provide a novel link between deubiquitination and growth arrest in OIS. Conversely, we found the ubiquitin E3 ligase (RNF13) to be up-regulated in OIS, suggesting that these proteins play an important role in ubiquitin-mediated proteasomal degradation in OIS.

In summary, the protein expression analysis revealed a lot of different processes regulated in OIS previously shown to be directly or indirectly linked to growth arrest, but also hinting at mechanisms yet to be further explored in the context of senescence.

Phosphoproteome Changes Specific for Senescence—Among the most interesting phosphorylation events specifically regulated in OIS are phosphoSTAT3, p53, BAZ1B, ephrin type-B receptor 2, and CD44. For Collin/p80 (COIL), total protein levels remained unchanged, but its phosphorylation at T303 was down-regulated in pre-OIS after only 3 days, and this was even more strongly and specifically pronounced in OIS after 9 days. Previously, Collin phosphorylation levels were shown to increase in mitosis (40), suggesting a role of Collin phosphorylation in growth inhibition in OIS.

Signal transducer and transcription activator 3 (STAT3) is a well-known mediator of cellular responses activated by several growth factors and cytokines. Tyrosine-phosphorylated STAT3 dimerizes to form an activated signal transducer that has been shown to be an important mediator in a whole variety of human cancers (41, 42). Moreover, it is interesting to note that inhibition of STAT3 activity also leads to the induction of pro-inflammatory cytokine expression, including IL-6 (43). In our study, we indeed observed down-regulation of STAT3 Tyr705 phosphorylation specifically in OIS, whereas its protein levels remained unchanged (supplemental Fig. S4, Fig. 4B). This suggests that STAT3 may be an important mediator of OIS that acts by reducing growth and inducing the inflammatory response needed to maintain the OIS signature, which needs to be investigated further. Another interesting phosphosite down-regulation was observed on p53 at Ser315.

Other phosphorylated proteins with sites specifically up-regulated in OIS included WSTF, a component of the chromatin remodeling complex, and the transmembrane proteins extracellular matrix receptor III (ECM-R/III/CD44) and ephrin type-B receptor 2 that mediate the communication between the ECM and adjacent cells (Fig. 2, supplemental Fig. S6). Especially for CD44, we identified many phosphorylation sites that were unchanged (Ser686, Ser706, and Thr720), down-regulated in both OIS and OISb (Ser718), or specifically up-regulated in OIS (Ser697). Ephrin type-B receptor 2 showed enhanced phosphorylation in OIS on T590 and S776, whereas T585 was down-regulated in both OIS and OISb.

DISCUSSION

In this work we report the most comprehensive dataset of protein and phosphosite regulations associated with the onset and maintenance of oncogene-induced senescence to date. Using Ti4+IMAC phosphopeptide enrichment and phosphotyrosine peptide immunoprecipitation, we were able to monitor a vast amount of signaling events. The high specificity and selectivity of Ti4+IMAC beads and pTyr antibodies allowed for the detection of important tumor suppressor (i.e. RB1, p53) and signaling proteins (i.e. STAT3, CDK1). This comprehensive high-quality dataset of OIS and its bypass contains many interesting observations that warrant further investigation in primary melanocytes and, perhaps, in vivo models to characterize the biological mechanisms involved.

We instigated OIS by ectopic expression of the BRAFV600E encoding oncogene in human primary fibroblasts. The expression of mutated BRAF led to substantial reprogramming of the proteome and phosphoproteome relative to cycling fibroblasts, both in the final OIS stage (9 days post-transfection) and in the early stage in which senescence was not yet apparent (3 days post-transfection) (Fig. 2). However, not all the observed changes can be attributed to the onset of senescence, as became apparent from our analysis of OISb cells, in which we introduced the mutant BRAF oncogene but rescued the cells from senescence with shRNA-mediated knock-down of the proinflammatory transcription factor C/EBPβ. The (phospho)proteome data also reveal that substantially less reprogramming of the proteome took place when comparing senescent (OIS) to bypassed nonsenescent (OISb) cells, after both 3 and 9 days (Fig. 2). Thus, BRAFV600E expression per se leads to substantial reprogramming of the proteome and phosphoproteome, and senescence-specific
changes are less numerous, indicating that senescence is a tightly regulated mechanism that can be altered by a small number of changes. That senescence is really evaded in OISb cells is made evident by several biomarkers that are differentially expressed between OIS and OISb, such as the relatively lower levels of interleukins (IL6, IL8) and SA-β galactosidase. These observations give credibility to our data, in that proteins and phosphosites found differentially regulated between OIS and OISb cells are potentially critical for onset and maintenance of OIS.

It has been shown previously that senescence can be induced by inhibition of the cell cycle via the p53-p21 pathway (44–46). We also found that BRAFV600E expression resulted in RB1 hypophosphorylation, up-regulation of p21, and repression of CDK signaling. When CDKs are activated by cyclins, their t-loop is repositioned, exposing the active site of CDK. An essential step in this activation is the phosphorylation of tyrosine 15 by Wee1 and Myt1 (47, 48). Interestingly, CDK1/2/3 Y15 phosphorylation was down-regulated strongly in OIS and to a lesser extent in OISb (Fig. 2B, Fig. 4B). Thus the down-regulation of CDK phosphorylation in OIS and OISb correlates well with the differentially elevated p21 levels, inhibiting CDK–cyclin complex formation. However, we did note that this mechanism was not specific for OIS, as p21 levels were enhanced and CDK protein and CDK target phosphorylation levels were repressed in both OIS and OISb cells (albeit to lesser extent in OISb). This suggests that these tumor-suppressive signals were not strong enough in the OISb cells to evade a full cell cycle arrest. However, we did note other differentially regulated proteins and sites that can be either directly or indirectly linked to OIS onset and maintenance. A summary of these OIS-specific events is displayed in Fig. 5.

A factor strongly and specifically present in OIS was FGF2, which was shown previously on oncogenic RAS-expressing cells to induce an irreversible cell cycle arrest that could be negated by a tyrosine kinase inhibitor (49). Despite the potentially different partners of RAS and RAF in oncogene-induced senescence and malignant transformation, a similar regulation of FGF2 in these studies calls for a more thorough investigation of a potential common mechanism.

Interestingly, the phosphorylation of the tumor suppressor protein p53 at Ser315 was specifically down-regulated in OIS cells (at 9 days) only (supplemental Fig. S5). This site was previously shown to be phosphorylated by two different kinases. Blaydes et al. demonstrated that CDK inhibition reduces Ser315 phosphorylation levels and that the p53 S315A mutant shows reduced transcriptional activity, resulting in lowered expression levels of p21 in cancer cell lines (50). However, in our data similar levels of p21 were observed in OIS and OISb, and in contrast to the observation in OIS, Ser315 phosphorylation was not down-regulated in OISb, which suggests that in our model p21 levels were independent of p53 Ser315 phosphorylation. The second kinase reported to phosphorylate p53 Ser315 is Aurora kinase A. Katayama et al. showed that phosphorylation of Ser315 by Aurora A leads to p53 ubiquitination and subsequent protein degradation (51). Therefore, reduced levels of S315 would suggest a greater stability of p53 in OIS cells than in cycling and OIS bypass cells, potentially leading to a prolonged p53 effect in OIS.

A potentially interesting phosphosite found up-regulated in OIS was Ser1342 of the tyrosine protein kinase WSTF (or BAZ1B), whose total protein levels remained similar in all conditions (Fig. 2, supplemental Fig. S6). WSTF is part of the chromatin remodeling complexes WICH (52) and WINAC (53) and was shown to tyrosine phosphorylate histone H2AX, thereby regulating the formation of γ-H2AX foci (54).
appearance of γ-H2AX foci is one of the hallmarks of senescent cells, and therefore specific up-regulation of phosphorylated WSTF could provide a novel link between foci formation and OIS.

A frequently observed feature of proteins and sites specifically regulated in OIS was their involvement in the extracellular matrix compartment that is known to play an important role in autocrine and paracrine cell signaling. The ECM can function as a sequestering binding site or a pool of growth factors that can be released upon ECM degradation (55). This is also the case for the ECM components heparin and heparan sulfate proteoglycans that can bind fibroblast growth factor 2, leading to enhanced receptor tyrosine kinase signaling (56, 57). Interestingly, proteins specifically regulated in the senescence phenotype contain ECM interacting (i.e. FGF2, CD44), ECM protein cleaving (i.e. MMP1, DPP4, THBD), ECM cleavage inhibitor (i.e. TIMP3, TFP12, SerpinB2/PAI-2), glycan processing (i.e. AGA, GLB1/b-galactosidase, PGMZL1), and membrane lipid processing proteins (i.e. GAA, PSAP). Although CD44, ephrin type-B receptor 2, and ICAM were extensively and differentially phosphorylated in OIS, not much is known about the effect of the specific phosphorylation sites on these ECM receptors. However, CD44 is known to bind to the glycosaminoglycan and ECM component hyaluronic acid and interact with matrix metalloproteinases (58) that we found to be regulated in OIS as well. Moreover, CD44 was also identified as an important cancer stem cell marker in a variety of different cancers (59, 60). Therefore, the role of CD44 in cell–ECM interaction and its differential phosphorylation status in senescence and cancer might be interesting to functionally validate. It is clear that a substantial amount of OIS-regulated proteins are acting on the extracellular space and matrix. Consistent with our data, other studies have shown that extracellular communication through protein secretion is the driving force behind the OIS phenotype (61–63). Therefore, functional studies on ECM regulation, organization, and ECM–receptor and ECM–growth factor interaction could further increase our understanding of OIS and cancer-related cell signaling in general.

In summary, this large dataset describes differential protein and phosphorylation changes upon oncogene transduction and oncogene-induced senescence, which sets the stage for function-based analysis of potentially novel tumor-suppressive mechanisms linked to OIS and cancer.

All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository (64) with the dataset identifiers PXD001068 and PXD000523.

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