Interferon-regulated suprabasin is essential for stress-induced stem-like cell conversion and therapy resistance of human malignancies

Sona Hubackova¹,², Miroslav Pribyl¹, Lenka Kyjacova¹,², Alena Moudra¹, Rastislav Dzijak¹, Barbora Salovska¹, Hynek Strnad³, Vojtech Tambor⁴, Terezie Imrichova¹, Jiri Svec⁵,⁶, Pavel Vodicka⁷, Radka Vaclavikova⁸, Lukas Rot⁹, Jiri Bartek¹,¹⁰,¹¹* and Zdenek Hodny¹*  

¹Laboratory of Genome Integrity, ³Laboratory of Genomics and Bioinformatics, and ⁵Laboratory of Cell and Developmental Biology, Institute of Molecular Genetics, v.v.i., Academy of Sciences of the Czech Republic, Prague 14220, Czech Republic  
²Molecular Therapy Group, Institute of Biotechnology of the Czech Academy of Sciences, BIOCEV, Vestec, Prague-West, Czech Republic  
⁴Biomedical Research Center, University Hospital Hradec Kralove, Sokolska 581, 500 05 Hradec Kralove, Czech Republic  
⁶Department of Radiotherapy and Oncology, Third Faculty of Medicine, Charles University, Srobarova 50, 10034 Prague 10, Czech Republic  
⁷Department of the Molecular Biology of Cancer, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague 14220 and Institute of Biology and Medical Genetics, 1st Medical Faculty, Charles University, 12000 Prague, Czech Republic

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/1878-0261.12480  
Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.  
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Correspondence to: Zdenek Hodny, MD, PhD; Department of Genome Integrity, Institute of Molecular Genetics ASCR, v.v.i., Videnska 1083, CZ 142 20 Prague 4, Czech Republic; Tel: (420)-24106 3151, Fax: (420)-24106 2289; E-mail: hodny@img.cas.cz and Jiri Bartek, MD, PhD; E-mail: jb@cancer.dk

Key words
5-azacytidine; therapy-resistance; suprabasin; cancer stem-like cells; interferon response

Abbreviations
5-AC, 5-aza-2′-deoxycytidine;
Akt, Protein kinase B;
Bcl XL, B-cell lymphoma-extra large;
Bim, Bcl-2-like protein 11;
CSCs, cancer stem cells;
DLL1, delta-like protein 1;
DLL4, delta-like protein 4;
EMT, epithelial-to-mesenchymal transition;
Erk, extracellular signal-regulated kinase;
ESCC, oesophageal squamous cell carcinoma;
fIR, fractionated irradiation;
GAPDH, glyceraldehyde 3-phosphate dehydrogenase;
GOBP, gene ontology biological process;
IFNβ, interferon beta;
IFNγ, interferon gamma;
IRF1, interferon responsible factor 1;
IRF7, interferon responsible factor 7;
ISG15, interferon-stimulated gene 15;
MAPK, mitogen-activated protein kinase;
MEK, MAPK/ERK kinase;
MX1, MX dynamin-like GTPase 1;
OCT4, octamer-binding transcription factor 4;
PCa, prostate cancer;
ROS, reactive oxygen species;
SBSN, suprabasin;
SILAC, stable isotope labeling with amino acids in cell culture;
SOCS1, suppressor of cytokine signalling 1;
Sox2, SRY (sex determining region Y)-box 2;
TGFβ, tumour growth factor beta;
TGFβR, tumour growth factor beta receptor.

Abstract

Radiation and chemotherapy represent standard-of-care cancer treatments. However, most patients eventually experience tumour recurrence, treatment failure and metastatic dissemination with fatal consequences. To elucidate the molecular mechanisms of resistance to radio- and chemo-therapy, we exposed human cancer cell lines (HeLa, MCF-7, and DU145) to clinically-relevant doses of 5-azacytidine or ionizing radiation and compared the transcript profiles of all surviving cell subpopulations, including low-adherent stem-like cells. Stress-mobilized low-adherent cell fractions differed from other survivors in terms of deregulation of hundreds of genes, including those involved in interferon response. Exposure of cancer cells to interferon-gamma but not interferon-beta resulted in the development of a heterogeneous, low-adherent fraction comprised of not only apoptotic/necrotic cells, but also live cells exhibiting active Notch signalling and expressing stem-cell markers. Chemical inhibition of MEK or siRNA-mediated knockdown of Erk1/2 and IRF1 prevented mobilization of the surviving low-adherent population, indicating that interferon-gamma-mediated loss of adhesion and anoikis resistance required an active Erk pathway interlinked.
with interferon signalling by transcription factor IRF1. Notably, a skin-specific protein suprabasin (SBSN), a recently identified oncoprotein, was among the top scoring genes upregulated in surviving low-adherent cancer cells induced by 5-azacytidine or irradiation. SBSN expression required the activity of the MEK/Erk pathway, and siRNA-mediated knockdown of SBSN suppressed the low-adherent fraction in irradiated, interferon-gamma- and 5-azacytidine-treated cells, respectively, implicating SBSN in genotoxic stress-induced phenotypic plasticity and stress resistance. Importantly, SBSN expression was observed in human clinical specimens of colon and ovarian carcinomas, as well as in circulating tumour cells and metastases of the 4T1 mouse model. The association of SBSN expression with progressive stages of cancer development indicates its role in cancer evolution and therapy resistance.

**Running title**
Role of suprabasin in cancer therapy resistance

1 **Introduction**

Over the last decades, genotoxic therapies including chemo- or radio-therapy have been the prevailing modalities used to treat cancer patients. Unfortunately, the initial effectiveness of radiotherapy and chemotherapy is commonly undermined by the primary and/or acquired resistance of tumour cells associated with the development of recurrent disease and progression towards largely incurable metastatic stage that is responsible for over 90% of cancer-related mortality. The mechanisms underlying the occurrence of cancer resistance to radiotherapy and chemotherapy are complex and still poorly understood. Accumulating evidence indicates that the resistance of cancer cells to radiotherapy and chemotherapy encompasses several different but partly interlinked mechanisms comprising therapy-induced molecular programs together with the initial intra-tumour genetic and epigenetic heterogeneity (Burrell et al., 2013; Kreso et al., 2013; Swanton, 2012).

Notably, recent research has provided evidence that the cellular response to genotoxic stress can contribute to phenotypic reprogramming of cancer cells into more resistant phenotypes. Such process commonly involves induction of epithelial-to-mesenchymal transition (EMT) and occurrence of cancer cells with stem-like characteristics (Ghisolfi et al., 2012; Gomez-Casal et al., 2013; Kyjacova et al., 2015; Skvortsova et al., 2008). Generally,
cancer-induced mesenchymal and stem-like cells appear to be more resistant to genotoxic stress than epithelial cancer cells (Blanpain et al., 2011). In addition, genotoxic therapies increase the migratory and invasive properties of malignant cells (Moncharmont et al., 2014) and alter the tumour-associated microenvironment, collectively promoting metastatic behavior of treatment-surviving cancer cells (Ruegg et al., 2011).

There is mounting evidence that EMT is coupled with activation of the stem cell program in both normal (Mani et al., 2008) and transformed cells (Han et al., 2013; Rhim et al., 2012). While cancer stem cells (CSCs) express EMT transcription factors such as Twist1, Snail and Slug, *vice versa*, EMT-undergoing cells were found enriched for CSC markers (Thiery et al., 2009). Related to the non-responsiveness of CSCs to the currently used treatment modalities, some authors detected putative CSCs following the therapy as the only residual cell population (Levina et al., 2008).

Suprabasin (SBSN) was originally found in suprabasal layer of murine and human keratinocytes and was described as an epidermal differentiation marker and component of keratinocyte cornified envelope and as a substrate for transglutaminase-mediated cross-linking (Alam et al., 2014; Park et al., 2002). However, its significant upregulation after increased demethylation of CpG islands in promoter of non-small cell lung carcinoma (Glazer et al., 2009) and salivary gland adenoid cystic carcinoma was described (Shao et al., 2012), and later experiments revealed a role of SBSN in tumour cell proliferation and invasiveness (Zhu et al., 2016). These studies collectively suggest that SBSN is a new candidate oncogene contributing to tumour development and progression. While Brother of the Regulator of Imprinted Sites (BORIS) factor contributes to regulation of SBSN expression (Gaykalova et al., 2012), detailed mechanistic insights into regulation of SBSN’s expression in tumours is still missing.

Recently, we have shown that exposure of human metastasis-derived prostate cancer (PCa) cell lines to a clinically relevant fractioned ionizing radiation (fIR) regimen resulted in development of three distinct fIR-surviving populations *in vitro* (Kyjacova et al., 2015): (i) senescent-like adherent cells with the potential to restart proliferation after the last dose of IR; (ii) low-adherent dormant cells with stem-like cell traits and preserved competence to re-attach and restore proliferation (*low-adherent cells*); and (iii) re-adherent cells derived from the low-adherent fraction with reverted epithelial features and retained tumourigenicity supporting a model of bidirectional interconversion in establishment of tumour cell heterogeneity (reviewed in (van Neerven et al., 2016)). We observed the involvement of an EMT-like program in the response of PCa cells to fIR with such phenotypic switch strongly.
dependent on the Erk1/2-mediated expression of Snail and associated with repression of E-cadherin. However, the basis for the heterogeneous response of cancer cells to genotoxic stress remains poorly understood.

In this study, we explored the signalling pathways involved in phenotypic remodelling of cancer cells induced by RT and/or CT. Comparing transcription and proteomic profiles of three human cancer models of interconverting subpopulations of stress-surviving tumour cells we identified a hierarchy of interferon and Erk signalling pathways in regulation of SBSN and its novel function in establishment of the phenotypic plasticity and heterogeneity of therapy-resistant cancer cell populations.

2 Material and Methods

2.1 Chemicals and antibodies

Acetone, phorbol 12-myristate 13-acetate (PMA), Ponceau S solution, 4′,6-diamidino-2-phenylindole (DAPI), MEK kinase inhibitor U0126, TGFβR1 inhibitor SB431542 and 5-azacytidine (5-AC) were purchased from Sigma (St. Louis, MO, USA). Human interferon gamma (IFNγ) and human interferon beta (IFNβ) were purchased from Peprotech (Rocky Hill, NJ, USA). MEK kinase inhibitor selumetinib (AZD6244) was purchased from Selleckchern (Munich, Germany).

For immunoblotting and indirect immunofluorescence, the following antibodies were used: rabbit monoclonal antibodies against Snail, Bcl-XL, Bim, Akt, Akt phosphorylated on serine 473, rabbit polyclonal antibodies against β-actin, Bim, SMAD2 phosphorylated on serine 465/467, STAT1 phosphorylated on tyrosine 701, Erk1 and mouse monoclonal Erk 1/2 (all from Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibody against GAPDH (GeneTex, Irvine, CA, USA), mouse monoclonal antibodies against E-cadherin and integrin α-2 (ITGA2; BD Biosciences, San Jose, CA, USA), rabbit polyclonal antibody against phosphothreonine 202/phosphotyrosine 204 of Erk1/2 (Promega, San Luis Obispo, CA, USA), rabbit polyclonal antibody against suprabasin (Abgent, San Diego, CA, USA), rabbit polyclonal antibody against suprabasin (Sigma, St. Louis, MO, USA), rabbit polyclonal antibodies against Erk2, IRF1 and IRF7 (all from Santa Cruz, Dallas, Texas, USA). Mouse monoclonal antibody against γ-tubulin was provided by Pavel Draber (Institute of Molecular Genetics, Prague, Czech Republic). IgG-HRP anti-rabbit (170-6515) and anti-mouse (170-6516) secondary antibodies produced in goat were purchased from Bio-Rad.
Laboratories (Hercules, CA, USA). Alexa Fluor 488 anti Rabbit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies for immunoblotting and indirect immunofluorescence were diluted 1 : 1000 (1 : 5000 for GAPDH) in 2.5% non-fat milk and 1 : 100 in 10% FBS/1 × PBS, respectively. Secondary antibodies for immunoblotting and indirect immunofluorescence were diluted 1 : 10 000 in 2.5% non-fat milk and 1 : 1200 in 10% FBS/1 × PBS, respectively.

2.2 Cell cultures

All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Human embryonic kidney cells HEK293, cervix adenocarcinoma HeLa, prostate carcinoma DU145, glioblastoma U373 and breast carcinoma MCF-7 cell lines were cultured in DMEM containing 4.5 g/l glucose (Biochrom, Berlin, Germany). HPV-16 E6/E7-transformed human mesenchymal stem cell line HS-5 and ovarian carcinoma SK-OV-3 cell lines were cultured in RPMI. All culture media were supplemented with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin sulphate (Sigma, St. Louis, MO, USA). RPMI medium was in addition supplemented with 1% non-essential amino acids (NEAA; Sigma Aldrich). Cells were kept at 37°C under 5% CO₂ atmosphere and 95% humidity.

DU145, MCF-7 and U373 cells were irradiated 24 hours after seeding (22 000 cells/cm²) with orthovoltage X-ray instrument T-200 (Wolf-Medizintechnik) using 0.5 Gy/min dose rate and thorium filter either daily with 10 doses 2 Gy or with a single dose of 2 or 10 Gy (Kyyjäjä et al., 2015). Half volume of culture medium was changed every 48 hours for fresh one. All low-adherent cells were collected by centrifugation (300 × g for 10 minutes) and returned back into cultivation flask. 24 hours after last dose of fIR and 72 hours after single dose low-adherent and adherent cells were harvested separately.

To treat cells with 5-AC, HeLa, HS-5 and MCF-7 cells were seeded at density 22 000 cells/cm² 24 hours before 5-AC addition. Stock solution of 5-AC in sterile water was added either directly into culture medium (day 1, 3, 5, 7) or with exchange of half volume of culture medium (day 2, 4 and 6) to final concentration 2 μM or 4 μM with or without MEK inhibitor selumetinib (1 μM). All low-adherent cells were always recollected as in case of fIR. Low-adherent and adherent cells were harvested separately at day 8. Alternatively, single dose of 5-AC was used and both low-adherent and adherent cells were harvested 72 hours later (see Figure 1a).

Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
To treat cells with interferons, HeLa or MCF-7 cells were seeded at density 22 000 cells/cm². Next day the culture medium was exchanged for fresh one with or without IFNγ (5 ng/ml) or IFNβ (50 ng/ml) and cells were incubated for additional 3 (HeLa) or 6 days (MCF-7). In case of Erk pathway inhibition, MEK inhibitor selumetinib (1 μM) was added together with the first addition of IFNγ and then every 24 hours either directly to culture medium or with medium exchange (half volume) made every 48 hours with recollection of all low-adherent cells as described above.

2.3 Human colon carcinoma and ovarian cancer samples

See Supplementary table 3 for patients characteristics. Informed written consent have been obtained from all patients in compliance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University, Prague, Ethical Commission of the National Institute of Public Health in Prague and by Ethics committee of the General University Hospital Prague.

2.4 4T1 mouse metastatic model

Six-week-old Balb/c mice (females) were injected with 1 × 10⁶ 4T1 cells in PBS into mammary gland. Mice were sacrificed after 3 weeks when tumours reached on average ± 800 mm³ and lungs, liver and blood were removed and processed according to protocol described by Pulaski et al. (Pulaski and Ostrand-Rosenberg, 2001). To determine the level of SBSN expression, 4T1 colonies selected by 6-thioguanine were harvested and processed as described below in section Quantitative real time PCR. Experiment was approved by the Czech academy of Sciences Ethics Committee and performed according to the Czech Council guidelines for the Care and Use of Animals in Research and Teaching.

2.5 Colony-forming assay

To estimate the potential of low-adherent cells to reestablish adherence, the modified colony-forming assay was used as reported (Kyjacova et al., 2015). Briefly, three days after the exposure to treatment, low-adherent cells were transferred into a new plate and left for 24 hours to allow adherence of residual mitotic cells. The remaining low-adherent cells were transferred to a new plate and allowed to re-adhere for 24 - 30 days. To visualize formation of colonies, cells were fixed at -20°C with 96% ice-cold methanol for 10 minutes and then stained with 0.5% crystal violet in ethanol at room temperature for 5 minutes.

Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
2.6 **Indirect immunofluorescence**

U373 cells were transfected by siRNA 24 hours after seeding at a density of 22,000 cells/cm² on glass coverslips. Culture medium was changed 24 hours after transfection. Cells were irradiated by a single dose of 10 Gy 48 hours post transfection. 72 hours post irradiation cells were fixed with 4% formaldehyde for 15 minutes and then permeabilized by 0.1% Triton X-100. After washing with PBS cells were incubated in 10% FBS/1 × PBS for 60 min, with primary antibody at room temperature for 1 hour, and after PBS wash with secondary antibodies at room temperature for 1 hour. After washing with 1 × PBS and ddH₂O, cells were stained with 1 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI, Sigma St. Louis, MO, USA), washed with ddH₂O and mounted by ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). Images were acquired by Leica DM6000 fluorescence microscope (Leica Microsystems).

Low-adherent MCF-7 cells were generated using single dose of 4 µM 5-AC. After 5 weeks with medium exchange every 3-4 days cells were washed with PBS and cyospun onto microscopic slides (400 × g for 5 minutes; Centurion Scientific, K3 Series, UK). Attached cells were fixed using 4% formaldehyde for 15 minutes and then permeabilized by 0.1% Triton X-100. After washing with PBS and ddH₂O, cells were stained with 1 µg/ml DAPI (Sigma St. Louis, MO, USA), washed with ddH₂O and mounted by ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). Images were acquired by Leica TCS SP8 confocal laser microscopy system (Leica Microsystems).

2.7 **Magnetic-activated cell sorting (MACS)**

Cells were treated for 72 hours and low-adherent cells were collected 24 hours after the last dose of the particular treatment. The AnnexinV-negative fraction of low-adherent human cancer cells was obtained by incubation of low-adherent cells with Dead Cell Removal MicroBeads (Dead Cell Removal Kit, Miltenyi Biotec, Germany) for 15 minutes and separated using magnetic field of an AutoMACS Pro magnetic separator (Miltenyi Biotec, Germany). The separated cells were processed immediately for immunoblotting, qRT-PCR or whole genome expression analysis.
2.8 Detection of apoptosis using fluorescence-activated cell sorting (FACS)

The numbers of viable and dead cells were analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Cells (adherent in one vial and dead plus low-adherent in other vial) were collected (400 × g at 4°C for 5 minutes), washed with PBS and stained for 15 minutes at RT with 0.3 µl of AnnexinV-Dyomics 647 in 100 µl of 1 × AnnexinV binding buffer (Apronex, Prague, Czech Republic). Immediately before analysis, 0.3 µl of Hoechst 33258 (10 mM, Invitrogen, Carlsbad, CA, USA) was added. The percentage of low-adherent cells (AnnexinV–/Hoechst–) was calculated according to formula low-adherent % = [(low-adherent cells / (low-adherent cells + live-adherent cells))] * 100. The calculated percentages of surviving low-adherent cells after the treatments were further normalized to the percentage of low-adherent cells in control conditions.

2.9 SDS-PAGE and immunoblotting

Cells were washed twice with PBS, harvested into Laemmli SDS sample lysis buffer (2% SDS, 50 mM Tris-Cl, 10% glycerol in double distilled H2O) and sonicated (3 × 15 seconds at 4 micron amplitude with 15 seconds cooling intervals) using Soniprep 150 (MSE, London, UK). Conditioned culture media were precipitated by acetone overnight, then samples were centrifuged (4000 × g, 10 minutes), air-dried pellets were dissolved into Laemmli SDS sample lysis buffer and sonicated (3 × 15 seconds at 4 micron amplitude with 15 seconds cooling intervals). Concentration of proteins was estimated by the BCA method (Pierce Biotechnology, IL, Rockford, USA). 100 mM DTT and 0.01% bromophenol blue were added to cell lysates before separation by SDS-PAGE. The same amount of protein (35 - 50 µg) was loaded into each well. Proteins were electrotransferred onto a nitrocellulose membrane using wet transfer and detected by specific antibodies combined with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, goat anti-mouse, Bio-Rad, Hercules, CA, USA). Peroxidase activity was detected by ECL (Pierce Biotechnology, Rockford, IL, USA) or SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH or γ-tubulin or were used as a marker of equal loading.

2.10 RNA interference

Specific siRNAs were introduced into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. 48 hours after lipofection cells were treated with fIR/IR, IFNγ and 5-AC. siRNAs were purchased from Thermo Fisher
Non-targeting siRNA sequences (Silencer® Select Negative Control No. 1, #4390843) were used as negative control siRNA (siNC). Sense sequences of used siRNAs are listed below: siErk1#1: 5´-GGA CCG GAU GUU AAC CUU Utt-3´, siErk1#2: 5´-UGA UGG AGA CUG ACC UGU Att-3´, siErk2#1: 5´-CAA CCA UCG AGC AAA UGA Att-3´, siErk2#2: 5´-GCA GAA AUG CUU UCU AAC Att-3´, siSBSN#1: 5´-AGA AGG UCA UUG AAG GGA Utt-3´, siSBSN#2: 5´-GGA GAA GGU UUU CAA CGG Att-3´, siIRF1#1: 5´-GCUCAGCUGUGCGAGUGUA-3´, siIRF1#2: 5´-CCU CUG AAG CUA CAA CAG Att-3´.

2.11 Quantitative real time PCR (RT-qPCR)

Total RNA samples were isolated using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) as described (Vlasakova et al., 2007). First strand cDNA was synthesized from 500 ng of total RNA with random hexamer primers using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA, USA). RT-qPCR was performed in ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix containing SYBR GreenE dye (Applied Biosystems, Foster City, CA, USA). The relative quantity of cDNA was estimated by the ΔΔCT method and data were normalized to RPL37a or β-actin (ACTB).

The following primers were purchased from Eastport (Prague, Czech Republic): hSBSN: 5´-CAG GCT GGA AAG GAA GTG GAG A-3´, 5´-CTT GAT GGC TGG AAG ATC CCG T-3´; Snail (SNAI1): 5´-TGC CCT CAA GAT GCA CAT CCG A-3´, 5´-GGG ACA GGA GAA GGG CTT CTC-3´; hSox2: 5´-CAA GAT GCA CAA CTC GGA GA-3´, 5´-GCT TAG CCT CGT CGA TGA AC-3´; CD44: 5´-CCA GGA GAA GCA GTG GTT TGG C-3´, 5´-ACT GTC CTC TGG GCT TGG GTG T-3´; p16INK4A (CDKN2A): 5´-CTC GTG CTG ATG CTA CTG AGG A-3´, 5´-GGT CGG CGC AGC AGT TGG GCT CC-3´; p21WAF1 (CDKN1A): 5´-TCA CTG TCT TGT ACC CTT GTG C-3´, 5´-GGC GTT TGG AGT GGT AGA AA-3´; DLL1: 5´-TGC CTG GAT GTG ATG AGC A-3´, 5´-ACA GCC TGG ATA GCG GAT ACA C-3´; DLL4: 5´-CTG CGA GAA GAA AGT GGA CAG G-3´, 5´-ACA GTC GCT GAC GTG GAG TTC A-3´; Hes1: 5´-GGA AAT GAC AGT GAA GCA CCT CC-3´, 5´-GAA GCG GTG CAT CTC GTT CAT G-3´; Hey1: 5´-TGT CGT AGC TGA GAA GGC TGG TGT-3´, 5´-TTG CCG TGA ACC ATC AGC AC-3´, 5´-ATT GAA ATC AGC CAG CAC GC-3´; OCT4: 5´-CAG CTT GGG CTC GAG AAG-3´, 5´-CCT CTC GTT GTG CAT AGT CG-3´; β-actin

Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
(ACTB): 5´-CCA ACC GCG AGA AGA TGA-3´, 5´-CCA GAG GCG TAC AGG GAT AG-3´; Herc5: 5´-CAA CTG GGA GAG CCT TGT GGT T-3´, 5´-CTG GAC CAG TTT GCT GAA AGT GG-3´; SOCS1: 5´-TTC GCC CTT AGC GTG AAG ATG G-3´, 5´-CTG CAT CTC TTG TAG CTT CAG A-3´; IRF1: 5´-CTG GCA CAT CCC AGT GGA A-3´, 5´-CAT CCT CAT CTG TTG TAG CTT CAG A-3´; IRF7: 5´-CCA CGC TAT ACC ATC TAC CTG G-3´, 5´-GCT GCT ATC CAG GGA AGA CAC A-3´; ISG15: 5´-CTG TGC TCC AGC AGC TCG AAG A-3´; MX1: 5´-GCC AGG AAA TGA ACA GGT TGC AG-3´, 5´-GCC AGG AAA TGA ACA GGT TGC AG-3´; mSBSN: 5´-CTC CCA CTC CCT GAA ATC TG-3´, 5´-GCC AGG AAA TGA ACA GGT TGC AG-3´, 5´-TAC CCG CCT TGA CCT GTA TGA G-3´; mSox2: 5´-TTC GGT GAC CTA GAC AGA GAA GC-3´, 5´-TTC GGT GAC CTA GAC AGA GAA GC-3´.

The data are expressed as the means ± S.D. of a minimum of three independent experiments performed in triplicates. The P-values were estimated using two-tailed Student’s t-test. p-values < 0.05 were considered as statistically significant.

2.12 Preparation of SBSN promoter-driven luciferase construct

The SBSN proximal promoter-driven luciferase reporter vector (pGL3-SBSN) was prepared by cloning a 2071 bp-long proximal promoter region upstream of the SBSN gene initiation codon (GRCh38.p12 chr19: 35,528,282 – 35,530,351) into a pGL3-Basic vector (Promega, San Luis Obispo, CA, USA) utilizing Gibson assembly. For this purpose, two pairs of primers were used: 1) pGL3-Basic FWD, 5´-TTC CCG ACC TTC CCA GCA ATC TCG AGA TCT GCG ATC TAA GTA AGC T-3´, pGL3-Basic REV: 5´-TTT TGG AGG GAG AGT CTC ACC CCG GGC TAG CAC GCG TAA G-3´, and 2) SBSN 2071 bp upstream FWD: 5´-CTT ACG CGT GCT AGC CCG GGG TGA GAC TCT CCC TCC AAA A-3´, SBSN 2071 bp upstream REV: 5´-CTT AGA TCG ATC TCG AGA TTG CTG GGA AGG TCG GGA A-3´. As templates for PCR, pGL3-Basic vector or MCF-7 genomic DNA isolated using Quick-DNA isolation kit (Zymo Research Corp., Irvine, CA) were used. Generated amplicons subsequently underwent multistep enzymatic reaction of Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), T5 exonuclease (New England Biolabs, Beverly, MA) and Taq DNA ligase (BioLabs). Sequencing control was performed using two primers LucNrev primer: 5´ - CCT TAT GCA GTT GCT CTC C-3´ and RVprimer3: 5´ -CTA GCA AAA TAG GCT GTC CC-3´.
2.13 Transient transfection and dual-luciferase reporter assay

HEK293 were seeded at a density of 22 000 cells/cm² onto 60 mm dish. 24 hours later the medium was exchanged and the cells were co-transfected with either 6 µg of pGL3-SBSN, pGL3-Control (SV40 promoter) or pGL3-Basic (promoterless) vector encoding Firefly luciferase together with 6 µg of pRL-TK vector encoding Renilla luciferase using the calcium phosphate method. After medium exchange 24 hours post transfection, cells were treated with IFNγ (5 ng/ml) or PMA (20 nM). MEK inhibitor selumetinib (1 µM) was added to cell cultures either alone or simultaneously with addition of IFNγ. Samples were collected 48 hours after treatments into 1 × Passive lysis buffer and further processed according to manufacturer’s protocol (Promega, San Luis Obispo, CA, USA). Luciferase activity was detected by 2104 EnVision Multimode plate reader (Perkin Elmer). Luminometric data of Firefly luciferase activity were normalized to Renilla luciferase activity and protein concentration determined by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA).

2.14 Whole-genome expression array

To obtain fIR-resistant cell fractions (10 times 2 Gy daily), DU145 and MCF-7 cells were seeded at density 14 000 cells/cm² into two 175 cm² cultivation flasks 24 hours before first dose. Low-adherent cells were recollected every medium exchange (48 hours). Low-adherent cells were separated from adherent ones 24 hours after last dose. One half of low-adherent cells was harvested (low-adherent fraction; see below) in parallel with adherent cells (adherent fraction), the remaining low-adherent cells were kept in culture to form readherent colonies and harvested after 21 days (re-adherent fraction). Proliferating cells served as controls (parental cells).

To generate 5-AC-resistant cell fractions (7 times daily dose of 5-AC), HeLa cells were seeded at density 14 000 cells/cm² into two 175 cm² cultivation flasks 24 hours before first addition of 5-AC (4 µM) and cultured as described above. 24 hours after the last dose of 5-AC one half of low-adherent cells was harvested in parallel with adherent cells. Readherent cells were prepared in similar manner like those for fIR. Proliferating cells served as control (parental fraction).
AnnexinV-negative (live) low-adherent cells were separated from dead cells by MACS as described above. Total RNA of all cell fractions (parental, low-adherent, adherent and re-adherent) was isolated using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). RNA integrity was assessed in Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA, USA). Total RNA was amplified using Illumina TotalPrep RNA Amplification Kit from Applied Biosystems (Foster City, CA, USA) according to the standard protocol, from a starting amount of 200 ng. cDNA quality and quantity were assessed in Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip. cDNA (750 ng) was hybridized, washed, and scanned according to the manufacturer’s instructions.

The raw expression data resulting from microarray were analyzed using the BeadArray package (Dunning et al., 2007) of Bioconductor within the R environment (R Development Core Team 2007). All hybridizations passed the quality control. The data were background-corrected and normalized with the probe level quantile method. Differential expression analysis was performed with the Limma package (Smyth et al., 2005) on intensities that were variance-stabilized by logarithmic transformation. Annotation provided by Bioconductor was used (illuminaHumanv4BeadID.db). To identify significantly perturbed pathways, we performed SPIA (Tarca et al., 2009) analysis on KEGG pathways: genes with \( P \)-values < 0.05 were considered as differentially transcribed. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6062.

2.15 Proteomic analysis

DU145 cells for proteomic analysis were cultivated in “heavy SILAC” low glucose medium (Sigma, D9443) supplemented with L-arginine-13C6,15N4 (84 mg/ml), L-lysine 13C6 (146 mg/ml), L-leucine (0.1 mg/ml), sodium pyruvate (1 mg/ml), D-glucose (25 mM) and 10% dialyzed FBS (Sigma, F0392). 95% efficiency of labelling was achieved after 10 population doublings in heavy media. Low-adherent cells were prepared according to previously published protocol (Kyjacova et al., 2015). Cells grown on T150 tissue culture flasks were irradiated with orthovoltage X-ray instrument T-200 (Wolf-Medizintechnik GmbH, St. Gangloff, Germany) using 0.5 Gy/min dose rate and thorium filter for 10 days with 2 Gy (fIR). Every third day medium was collected and centrifuged. Pelleted cells were resuspended in fresh medium and returned to the original dish. After the last dose, the medium containing low-adherent cells was transferred to a fresh flask and incubated for additional 24 hours.
allow the mitotic cells to re-adhere. Medium was centrifuged at 300 × g for 10 minutes to pellet the low-adherent cells. AnnexinV-negative fraction, was obtained by incubation with Dead Cell Removal MicroBeads (Dead Cell Removal Kit, Miltenyi Biotec, Germany) for 15 minutes and separation in magnetic field of AutoMACS Pro magnetic separator (Miltenyi Biotec).

Cells from three separations were lysed in lysis buffer (100 mM 4-ethylmorpholine pH 8.3, 10% acetonitrile, 1% w/v sodium deoxycholate). Lysates were heated to 95°C for 5 minutes and sonicated until clear, non-viscous solution was obtained. Total protein concentration was measured by BCA assay (Thermo, cat No 23225). Lysates from heavy labelled cells (floating population) and nonlabelled cells (adherent non-irradiated cells) were mixed in 1 : 1 ratio to obtain total 60 µg of protein. Samples were reduced with neutralized 5 mM TCEP (Thermo, 77720) at 60°C for 15 minutes followed by alkylation of cysteines with 10 mM MMTS, at room temperature for 30 minutes. Proteins were digested with trypsin GOLD (Promega, V5280 ) at 1 : 40 ratio (enzyme : protein) at 37°C for 16 hours.

Digested proteins were acidified by addition of 4 µl of 40% TFA. Precipitated deoxycholate was extracted five times with ethyl acetate. Insoluble particles were spun at 16000 × g for 15 minutes and the supernatant was dried using speed vac.

Peptides were desalted using Empore C18 SPE cartridges. Peptide samples were fractionated offline using Waters XBridge Peptide BEH C18 (2.1 µm × 150 mm) by a linear gradient of mobile phase (A: 20 mM ammonium formate/2% acetonitrile; B: 20 mM ammonium formate/80% acetonitrile) into 42 fractions, which were then combined into 8 final fractions for analysis. 5 µl from each pooled fraction was loaded on Acclaim PepMap100 nanoViper trapcolumn (C18, 3 µm, 100 Å, 75 µm i.d. × 2 cm, Thermo) for 3 minutes using flow rate 8 µl/min. Peptides were separated on Acclaim PepMap RSLC column (C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm, Thermo) in flow rate 200 nl/min and eluted in 240 min gradient (A- 2:98 acetonitrile/water/0.1% formic acid; B 80 : 20 acetonitrile/water/0.1% formic acid) and analysed online using Q-Exactive hybrid quadrupole mass spectrometer (Thermo).

Raw data were processed using MaxQuant (Cox and Mann, 2008). Statistical analysis and annotation enrichment analyses were performed in Perseus software (Tyanova et al., 2016).
2.16 Data processing and statistical analysis

Unless stated otherwise, data are mean values ± SEM of at least three independent experiments. In mouse experiments, groups of 6 animals were used, unless stated otherwise. Two-way Anova presented as mean ± standard error of means (SEM) was used to assess statistical significance with \( p < 0.05 \) being regarded as significant, using GraphPad Prism software. Images are representative of at least three independent experiments. FACS data were analyzed using FlowJo 9.6.4 cytometric analytical software (Tree Star, Stanford University, USA).

3 Results

3.1 5-Azacytidine induces loss of adhesion and stem cell traits associated with resistance to anoikis

The exposure of human cancer cells to fractioned ionizing radiation (fIR) resulted in development of resistant cell subpopulations including cells losing adhesion and bearing cancer stem cell-like features (Kyjacova et al., 2015). To find out whether the development of such low-adherent cell fraction represents a common response of tumour cells to genotoxic stress, we exposed human cancer cell lines HeLa and MCF-7, and retrovirally immortalized bone marrow (BM) mesenchymal stem cells HS-5 to 5-azacytidine (5-AC), a hypomethylating agent currently used in therapy of high-risk myelodysplastic syndrome (MDS; (Kornblith et al., 2002)) and tested in numerous clinical trials for treatment of solid malignancies. We used two modes of 5-AC exposure (as specified for each experiment in figure legends): 1) a 'clinical-mimicking' regimen of 5-AC with daily administration for seven consecutive days with cells harvested at day 8 (see Figure 1a left), and 2) a single dose of 5-AC with harvest at day 3 showing a similar effect on resistance development achieved in shorter time (see Figure 1a right), both within the concentration range of 5-AC that is reached in the humans (in MDS patients 5-AC is administered at a dose of 75 mg/m\(^2\)/day; (Marcucci et al., 2005; Rudek et al., 2005). Both 5-AC-treatments mostly reproduced the effects of fIR published previously (Kyjacova et al., 2015) showing equivalence of both 5-AC treatments. Besides senescence-like state (not shown) and cell death (see Supplementary Figure S1a), 5-AC treatment induced development of a low-adherent cell subpopulation (see Supplementary Figure S1b) the onset of which was detectable in all three cell types from day 2 onwards (see Figure 1b). Comparable to fIR, 5-AC-induced occurrence of anoikis-resistant cells that survived in low-adherent (dormant) state for 2 - 4 weeks before readhesion and renewal of proliferation (Supplementary Figure S1c; only MCF-7 cells shown). The low-adherent cells
exhibited stem cell-like traits such as elevation of Sox2, CD44, or OCT4 transcripts (see Figure 1c for Hela cells and Supplementary Figure S1d and e for MCF-7 and HS-5 cells), activation of Notch signalling (detected as elevation of Notch ligands DLL1 and DLL4 and Notch effector transcription factors Hes1 and Hey1; see Figure 1d for Hela cells and Supplementary Figure S1f and g for MCF-7 and HS-5 cells) and elevation of Snail (see Figure 1e and Supplementary Figure S1h for protein and mRNA levels of Snail, respectively). Furthermore, the lack of proliferation in the low-adherent cell fraction correlated with increased mRNA levels of inhibitors of cyclin-dependent kinases p16\(^{\text{INK4a}}\) (p16) and p21\(^{\text{waf1}}\) (p21; Figure 1f for Hela cells and Supplementary Figure S1i and j for MCF-7 and HS-5 cells; note, the mRNA levels of cell cycle cyclin-dependent kinases were significantly downregulated as detected by transcriptome analysis). In addition, the level of antiapoptotic factor Bcl-XL was most pronounced in the low-adherent fraction (Figure 1g). Together with suppression of protein levels of E-cadherin and integrin \(\alpha V\) (Supplementary Figure S1k) this all demonstrated that 5-AC is capable of inducing phenotypic changes reminiscent of those evoked by fIR (Kyjacova et al., 2015). The activation of kinases Erk1/2 (detected as phosphothreonine 202 and phosphotyrosine 204 of Erk1 and Erk2, respectively) and Akt (detected as phosphoserine 473) was observed (Supplementary Figure S1l). Consistent with a previous study (Kyjacova et al., 2015), inhibition of Erk1/2 activity by MEK inhibitor selumetinib suppressed survival of the low-adherent fraction and reconstitution of fully adherent colonies (Supplementary Figure S1m). Of note, siRNA-mediated knockdown of Bim, a protein involved in anoikis, whose degradation is controlled by Erk activity, resulted in preservation of viability in low-adherent cells even in the presence of selumetinib (Figure 1h; for efficiency of Bim downregulation, see Figure 1i), supporting the role of the Erk/Bim pathway in the survival of low-adherent cells.

Altogether, these data demonstrate that 5-AC induces phenotypic plasticity characterized by survival of low-adherent tumour cells with stem cell-like features in different human cell types in a manner similar to fIR, thus indicating that this type of cell response is shared by various genotoxic insults.
3.2 Elevated interferon response is a common feature of surviving low-adherent cells

To further characterize the low-adherent cells surviving 5-AC and radiation treatments, a comparative analysis of gene expression profiles of irradiated cells (DU145 and MCF-7; 10 times 2 Gy) with 5-AC-treated cells (HeLa; 7 times daily dose of 4 μM 5-AC) was performed using Illumina human gene expression array. The hierarchical clustering of resistant populations revealed 2449 commonly deregulated protein-coding genes in low-adherent survivors (Figure 2a and Supplementary Table S1a). The heat map representation of the clustered matrix showed enrichment of genes involved in the innate immune response pathways (cluster I), specifically, NF-κB, Toll-like receptor, and interferon signalling (for details, see Figure 2b, Supplementary Figure S2a and Supplementary Table S1b). The changes of mRNA levels of several interferon stimulated genes (IRF1, IRF7, ISG15, Herc5, SOCS-1, and MX-1) were validated by RT-qPCR in HeLa cells treated with 5-AC for seven days (Figure 2c). Cluster II predominantly contained genes involved in the mitochondrial biogenesis and maintenance. Finally, cluster III contained genes involved in the regulation of transcription. These genes were, conversely, upregulated in all stress-induced low-adherent cells in comparison to the non-treated controls (Supplementary Table S1b).

To obtain a more comprehensive view of the biological state of low-adherent cells, we performed proteomic profiling of irradiated (10 times 2 Gy) DU145 cells using SILAC and mass spectrometry ((Ong et al., 2002); Supplementary Table S2a). As a next step, the protein expression data were correlated with mRNA expression profiles of fIR low-adherent DU145 cells using 2D annotation enrichment ((Cox and Mann, 2012); Supplementary Figure S2b and Supplementary Table S2b). Analogous to gene expression profiles, the proteomic pattern in the fIR low-adherent DU145 cells was dominated by the 'interferon response' pathway including proteins encoded by interferon-stimulated genes (ISGs) such as HLA-C, OAS1 and OAS3, ISG15 and ISG20, MX1, and STAT1 ((Der et al., 1998); Supplementary Figure S2c; for relative expressions of genes containing this annotation see Figure 2d).

To conclude, both fIR- and 5-AC-treatment induced development of the low-adherent surviving cell fraction characterized by profound changes in the expression of genes and proteins involved mainly in interferon response and cell cycle regulation.
3.3 **IFNγ but not IFNβ induces loss of adhesion and anoikis-resistant survival of human cancer cells**

Based on the transcriptome and proteomic profiling, we next tested whether the activity of the interferon pathway is causally involved in the development of low-adherent state and survival of cancer cells. For this purpose, HeLa and MCF-7 cells were exposed to daily administration of interferon type I (IFNβ) and type II (IFNγ) for three or six days, and the development of the low-adherent cell subpopulation was followed. In contrast to IFNβ, IFNγ significantly induced low-adherent cell population from day 3 in HeLa and day 6 in MCF-7 cells (Figure 3a). Notably, the administration of IFNγ induced the loss of adhesion also in HS-5 cells (Supplementary Figure S3a). As expected, besides STAT1 receptor-mediated phosphorylation (detected as STAT1 tyrosin 701 phosphorylation), the response to IFNβ and IFNγ was reflected by induction of transcription factors involved in the late interferon response IRF7 and IRF1, respectively (see Figure 3b for HeLa and 3c for MCF-7 cells).

Formation of large extracellular vesicles (see Supplementary Figure S3b; (Rak, 2010)) in both HeLa and MCF-7 cells was associated with a switch to the amoeboid-like form of cell migration (Di Vizio et al., 2009) of low-adherent cells (as shown in Supplementary Video S1). Interestingly, some IFNγ-induced low-adherent cells were able to re-adhere within several days after the end of the treatment and to reestablish adherent cell growth (Figure 3h, left). Similarly to fIR, the extended exposure of cells to IFNγ resulted in marked elevation of Sox2 and Oct4 mRNA levels in the low-adherent cell fraction (Figure 3d; HeLa cells shown) and activation of Erk1/2 (see Figure 3e for HeLa and Figure 3f for MCF-7 cells). Intriguingly, MEK inhibitor selumetinib (Supplementary Figure S3c) suppressed the IFNγ-induced development of low-adherent cells (Figure 3g) as well as the establishment of re-adherence (see Figure 3h, right), indicating crosstalk between IFNγ and MEK/Erk signalling pathways in the anoikis-resistant cancer cell survival. Down-regulation of late interferon response transcription factor IRF1 by RNA interference (Supplementary Figure S3d and e) resulted in suppression of the IFNγ-induced (Figure 3i) as well as IR-induced (Figure 3j) low-adherent cell fraction, supporting the causal role of IFNγ signalling in low-adherent survival after genotoxic stress.
This data shows that prolonged activation of the IFNγ/JAK signalling pathway in human cancer cell models grown in vitro induces changes in cell adhesive properties and development of the low-adherent cell subpopulation matching phenotypic reprogramming observed after fIR and 5-AC administration, respectively. Altogether, these findings indicate that the development of radioresistant cell subpopulations is mediated by the concerted action of the IFNγ/JAK and Erk signalling pathways.

3.4 Upregulation of suprabasin is a component of the shared response to stress induced by 5-azacytidine, fIR, and IFNγ

Furthermore, comparative analysis of transcript profiles of low-adherent cells induced by fIR (DU145 and MCF-7) and 5-AC (HeLa) revealed 463 genes whose transcripts were altered in the same direction (see Venn diagram in Supplementary Figure S4a). Notably, among the top scoring genes upregulated in surviving low-adherent cell fractions was suprabasin (SBSN; Supplementary Figure S4b), an oncoprotein associated with tumour progression and metastasis (Glazer et al., 2009; Shao et al., 2012; Zhu et al., 2016). Indeed, the elevation of SBSN transcript levels was confirmed independently by RT-qPCR in adherent and low-adherent fractions of all tested models including IFNγ-treated HeLa cells (Figure 4a-c). Notably, the protein levels of SBSN isoform 1 (61 kDa) were markedly elevated specifically in low-adherent fractions (see Figure 4d-f). Intriguingly, RNA interference-mediated downregulation of SBSN (Supplementary Figure S4c and d) suppressed the development of low-adherent cell fraction induced both by IFNγ (Figure 4g; HeLa cells shown) and 5-AC (Figure 4h; HeLa and MCF-7 cells shown).

As SBSN expression is associated with some human malignancies, we searched for cancer cell lines expressing the SBSN under unperturbed conditions. SBSN expression was not detected in most cell types tested (DU145, LNCaP, 22RV1, PC-3, MCF-10a, MCF-7, MDA-MB-231, BT474, ZR-75-30, MRC-5, H1299, HS913T, NCI-H28, NCM 460, HCT166, HT29, A375, HS-5, HEK293T, Panc-1, U2OS, HeLa, hTert RPE-1, IMR90, and BJ cells; not shown), however, the SBSN isoforms 2 and 3 protein levels were detected in cell lysates and conditioned media of human glioblastoma U373 and ovarian carcinoma SK-OV-3 cell lines (see Supplementary Figure S4e and f for immunoblotting, and Supplementary Figure S4g and h for indirect immunofluorescence).
To further examine the functional role of SBSN in radioresistance, the effect of SBSN downregulation on radiosensitivity of the U373 cell line was estimated. Indeed, knock down of SBSN suppressed the development of IR-induced (2 Gy) low-adherent cell fraction (Figure 4i) and increased the percentage of apoptotic cells (Supplementary Figure 4i) supporting the functional role of SBSN in radioresistance of glioblastoma cells.

Altogether, our findings show that the expression of SBSN is a shared feature of the cell response to genotoxic stress induced by different stimuli and indicate that SBSN is required for the stress-induced phenotypic plasticity, cellular heterogeneity and resistance to apoptosis of tumour cell populations.

3.5 IFNγ- and 5-AC-induced expression of SBSN is mediated by the MEK/Erk pathway

Next we examined how is the SBSN gene regulated by IFNγ and other stresses. As shown in Figure 5a, RNA interference-mediated knock down of IRF1 resulted in suppression of IFNγ-induced elevation of the SBSN transcript in HeLa cells. As IFNγ activated Erk1/2 (Figure 3e and 3f) and MEK inhibitors affected IFNγ-induced development of low-adherent fraction (Figure 3g), we examined whether SBSN is regulated by IFNγ-induced activation of Erk signalling. Down regulation of IRF1 suppressed IFNγ-induced activity of Erk1/2 (Figure 5b) and treatment of IFNγ-exposed HeLa cells with selumetinib resulted in suppression of the IFNγ-induced upregulation of SBSN (Figure 5c) to a degree comparable with the impact of Erk1/2 down regulation (Figure 5e; see Supplementary Figure S5a for efficiency of Erk1/2 down regulation), indicating that Erk activity contributes to the observed upregulation of SBSN gene expression mediated by IFNγ. The effect of inhibition of the Erk pathway (Supplementary Figure S5b) on the mRNA expression of SBSN was also found in HeLa and MCF-7 cells exposed both to 5-AC and selumetinib (Figure 5d and Supplementary Figure S5c and S5d; note MEK inhibitor U0126 was used to further support the data). Additionally, the down regulation of Erk1 and/or Erk2 also suppressed 5-AC-induced elevation of SBSN mRNA (see Figure 5f; for Erk1/2 down regulation efficiency, see Supplementary Figure S5e), suggesting again the role of the Erk pathway in stress-induced SBSN expression.

Furthermore, a luciferase activity driven by the proximal regulatory region of the SBSN gene spanning nucleotides –2000 to +71 relative to the SBSN transcription start site (pGL3-SBSN) was estimated in HEK293 cells treated either by IFNγ, MEK inhibitor or both (see Material Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
and Methods). Indeed, IFNγ treatment induced significantly *Firefly* luciferase expression driven by the SBSN proximal promoter relative to a promoterless construct (pGL3-Basic), whereas the MEK inhibitor selumetinib almost completely abolished it (Figure 5g) suggesting the effect of IFNγ on SBSN gene expression is mediated by Erk1/2 signalling. Moreover, an activator of MAPK signalling phorbol 12-myristate 13-acetate (PMA) induced SBSN promoter-driven *Firefly* luciferase expression further supporting transcriptional control of SBSN by Erk1/2 in accordance with a previous study (Park et al., 2002).

Collectively, these findings indicate the crucial role of Erk kinase signalling in the upregulation of SBSN expression in cells exposed to genotoxic stress.

### 3.6 SBSN is expressed in malignant human tissues and is associated with metastasis

Recent reports indicate SBSN expression in human malignancies such as glioblastoma (Formolo et al., 2011) or lung cancer (Glazer et al., 2009). Indeed, the analysis of SBSN expression in colorectal cancer biopsies (n = 8) provided similar results (Figure 6a). While normal mucosa surrounding the tumour tissue was always negative for SBSN mRNA expression, all tumour samples were positive (p < 0.05). Similarly, the SBSN mRNA levels in ovarian carcinoma biopsies (Figure 6b; n = 100) were significantly higher compared to non-malignant ovary tissue (n = 14, p < 0.0001).

Due to association of SBSN expression with EMT programme and cell stemness observed in our *in vitro* experiments, we next examined whether the SBSN expression is associated with development of metastases *in vivo*. Using a mouse model of 6-thioguanine-resistant 4T1 cells that frequently metastatize to lungs and liver, we compared SBSN transcript levels in parental cells, primary tumours, 4T1-derived circulating tumour cells (CTCs) and lung and liver metastases (see Material and Methods for details). Notably, the level of SBSN expression was significantly higher in CTCs and lung and liver metastases compared to primary tumour and parental 4T1 cells (Figure 6c), a feature that correlated with increased Sox2 expression in these cells (Figure 6d).
Altogether, SBSN is expressed in fully developed human malignant lesions of colorectal and ovarian carcinomas. Furthermore, high expression of SBSN in circulating tumour cells and metastatic lesions detected in our mouse model suggests association of SBSN with the metastatic process.

4 Discussion
Metastatic dissemination frequently combined with development of resistance to current antitumour therapies are the most serious complications of malignant diseases and cause more than 90% of deaths among cancer patients. The aim of this study was to elucidate the mechanisms of acquired resistance to radiotherapy and chemotherapy in several human cancer cell models, with emphasis on better understanding the development of a subpopulation of cancer cells characterized by anoikis-resistant survival. Among many deregulated genes detected by cDNA microarray analysis of transcript profiles from anoikis-resistant human tumour cells induced by 5-azacytidine or fractioned ionizing radiation, we identified elevation of gene transcripts regulated by the interferon signalling pathway and IFNγ itself (but not IFNβ) as being capable of inducing the anoikis-resistant state. Furthermore, we pinpointed SBSN, the recently reported oncoprotein, as an effector factor involved in the development of cancer cell resistance phenotypes. Elevated expression of SBSN in mouse circulating tumour cells and metastases, as well as clinical samples of human colorectal and ovarian carcinomas, supported our in vitro findings of SBSN being involved in cancer development and progression.

Interferons are known to induce genotoxic stress (see (Hubackova et al., 2015) and references herein). Here we observed that the exposure of tumour cells to IFNγ but not IFNβ was sufficient to mobilize a heterogeneous low-adherent and floating matter composed mainly of apoptotic and necrotic cell bodies and a small fraction of cells that survived the IFNγ-induced stress and later re-established adherent cell growth. These results indicate that interferon response itself or responses triggered by genotoxic insults alter cell adhesion properties in a subpopulation of cancer cells that is also associated with resistance to anoikis resulting in increased invasiveness of these cells.
As was shown previously (Kyjacova et al., 2015) low-adherent cells with stem cell-like features are continuously generated from the adherent, senescent phenotype-developing, fraction of resistant cells. Although the low-adherent cells share partially their gene expression pattern with senescent cells, for example elevated p21\(^{waf1}\) and p16\(^{INK4a}\) and several SASP factors, our comparison of global transcription profiles of low-adherent versus adherent cells indicates they are not identical. The major functional difference between the low-adherent and adherent cell fraction is evident from the ability of the former cells to re-attach and re-enter the cell cycle after few weeks. This correlates with the recently published findings that a reprogrammed gene expression profile associated with dedifferentiation, stem cell-like features, EMT, increased levels of c-myc and STATs, that we observed also in our low-adherent cells, are responsible for senescence bypass and therapy resistance (Deschênes-Simard et al.). Furthermore, given that cytoplasmic DNA elicits IFN responses (primarily type I IFNbela) and NFkappaB signalling through the cGAS/STING pathway (Sun et al., 2013), we also searched the transcriptomes of the anoikis-resistant cell subpopulation for evidence of IFNbela and NFkappaB activity. Indeed, our data show increased mRNA levels of IFNB1, IL1, TNF, IL6, and IL18, common targets of the cGAS/STING pathway. Future studies should address the possibility that the cGAS/STING pathway may contribute to development of the low-adherent phenotype and thereby play a role in survival and radiotherapy-resistance of cancer cells.

Our analysis of IFN\(\gamma\)-induced elevation of SBSN revealed that activation of Erk1/2 is a prerequisite for SBSN expression reminiscent of endothelial cells treated with EGF as an activator of Erk pathway (Alam et al., 2014). Treatment of cells with IFN\(\gamma\) resulted in increased Erk1/2 activity, the inhibition of which by MEK inhibitors reduced both SBSN expression and occurrence of low-adherent population after either fIR, 5-AC or IFN\(\gamma\). Although we did not explore the mechanism of Erk activation by IFN\(\gamma\) in depth, several reports show the ability of IFN\(\gamma\) to activate the Erk pathway (see, e.g. (Cho et al., 2007; Halfter et al., 2005; Harris et al., 2007; Sakatsume et al., 1998; Stancato et al., 1998)). Upon IFN\(\gamma\) stimulation of keratinocytes, suppressor of cytokine signalling 1 (SOCS1) induced by JAK/STAT pathway inhibited IFN\(\gamma\) receptor and STAT1 phosphorylation but maintained Erk activation (Madonna et al., 2008). Together with our observation of increased expression of SOCS1 in the low-adherent cells (Figure 2c) these results implicate SOCS1 in modulation of therapy resistance via stabilization of Erk signalling.
While the exact molecular function of SBSN remains currently unknown, there is emerging evidence that aberrant expression of SBSN is associated with human malignancies. SBSN has been found among genes co-ordinately expressed in non-small cell lung carcinoma characterized by promoter hypomethylation (Glazer et al., 2009). Intriguingly, ectopic expression of SBSN in some lung squamous cell carcinoma cell lines and normal lung fibroblasts is associated with growth advantage (Glazer et al., 2009). Likewise, SBSN promotes proliferation of oesophageal squamous cell carcinoma (ESCC) cell lines *in vitro* (Zhu et al., 2016). In addition, SBSN expression correlated with ESCC progression (i.e., SBSN expression correlated positively with increasing clinical tumour staging and negatively with overall patients’ survival time (Zhu et al., 2016)). Furthermore, SBSN was found as part of the secretome of highly invasive glioblastoma cell lines (Formolo et al., 2011). Notably, aberrant expression of SBSN was found in pleural effusions of lung adenocarcinoma patients (Sheng and Zhu, 2014) and in tumour endothelial cells of renal and colon carcinomas (Alam et al., 2014). These recent findings of cancer-associated expression of SBSN were further supported and extended in our present study by detection of SBSN in colorectal and ovarian carcinomas.

The presence of SBSN transcript in both adherent and anoikis-resistant low-adherent cell populations, yet the protein present predominantly in the latter cell fraction, suggested a potentially novel role of SBSN in the development of low-adherent state and anoikis-resistant survival. This scenario was indeed supported by the experiments manipulating SBSN levels, where the expression of SBSN dictated the amount of cells in the low-adherent state. Suprabasin overexpression activates the Wnt/β-catenin signalling pathway (Zhu et al., 2016), which has been implicated as a niche factor to maintain stem cells in a self-renewing state (Nusse, 2008). Wnt/β-catenin signalling is often associated with EMT as an activator of EMT transcription factors Zeb, Snail or Slug (Medici et al., 2008; Sanchez-Tillo et al., 2011). Thus, expression of SBSN in low-adherent cells can be responsible both for EMT due to increased expression of Snail and Zeb2 (Figure 1e and Supplementary Figure S1g), and for induction of cancer stemness (Figure 1c and Supplementary Figure S1c, d).

Based on these findings we propose the role of SBSN in stress resistance of cancer cells. It should be emphasized that SBSN may be involved in the maintenance of tumour invasiveness and metastatic potential as was reported for salivary gland adenoid cystic carcinoma (Shao et al., 2012). The association of SBSN expression with tumour aggressiveness is underscored by Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
our data indicating that SBSN induction is highest in circulating mouse tumour cells and metastatic deposits. Collectively, these findings implicate SBSN in promoting the aggressive phenotype of tumour cells.

Notably, Aibara et al. identified anti-suprabasin antigen activity in immune complexes present in cerebrospinal fluid of patients with the neuropsychiatric form of systemic lupus erythematosus (Aibara et al., 2018). This example of SBSN immunogenicity, documented by autoantibodies reactive with astrocytes causing an inflammatory response, may have implications for future attempts to apply immunotherapy against SBSN-expressing human malignancies. Consistent with the presence of SBSN expression in brains of newborn mice (Park et al., 2002) our data indicate that SBSN can be expressed under certain conditions also in cells other than the suprabasal layer of the epidermis. From this perspective, SBSN may serve as a tumour neo-antigen to be exploited in anti-cancer immunotherapy, particularly for tumours resistant to standard-of-care treatment modalities such as radiation used in our present study.

5 Conclusions

In conclusion, our findings indicate that genotoxic stresses accompanying radiotherapy or chemotherapy can induce plastic changes of the cancer cell phenotype and increased heterogeneity of surviving cancer cell populations. We showed that survival of a subset of cancer cells in a low-adherent state depends on the concerted activity of IFNγ and Erk signalling pathways activated by radiation- or chemically-induced stress (Figure 5g). Furthermore, the expression of suprabasin in such low-adherent compartment of radio- and chemo-resistant cells suggests an unorthodox mechanism through which cancer cells can respond to genotoxic stress. Notably, our data highlight the dependency on SBSN as a potential vulnerability of aggressive and metastatic tumours that are commonly refractory to current standard-of-care treatments.

Authors contributions

SH, MP and LK: Design, realization and analysis of experiments, manuscript writing. AM, RD and TI: Realization and analysis of experiments. BS and VT: Proteome realization and analysis. HS: Transcriptome realization and analysis. JS, PV, RV and LR: Patient sample
collection and preparation. JB and ZH: Design and analysis of experiments, manuscript writing.

Acknowledgements
This study was supported by Institutional Grant (Project No. RVO 68378050), Grant Agency of the Czech Republic (Project No. 17-07635S), DiaNa21 (Smartbrain, s.r.o.), the Danish Council for Independent Research (grant DFF-4181-00506), the Swedish Research Council (VR-MH 2014-46602-117891-30), the Danish National Research Foundation (Project CARD, DNRF125), the Novo Nordisk Foundation (grant 16584), BIOCEV European Regional Development Fund CZ.1.05/1.100/02.0109 and Institute of Biotechnology (RVO: 86652036) and European Regional Development Fund - Project ENOCH/CZ.02.1.01/0.0/0.0/16_019/0000868. RV was supported by "Center of clinical and experimental liver surgery" UNCE/MED/006. MP was supported in part by the Faculty of Science, Charles University, Prague. We acknowledge to Dr. P. Skapa (Department of Pathology and Molecular Medicine, Second Faculty of Medicine and Motol University Hospital, Charles University in Prague, Czech Republic) for histopathological verification of ovarian tumor tissue samples. We acknowledge R. Liska and S. Pavelka for the help with cell irradiation, E. Davidova for the help with in vivo experiments, T. Vomastek for the anti-Erk2 antibody, and Pavel Draber for the anti-γ-tubulin antibody. We are grateful to M. Vancurova for excellent technical assistance.

Conflict of Interest
We confirm that the data presented in the manuscript are novel, they have not been published, and are not under consideration for publication elsewhere. The authors declare that they have no conflict of interest.

References
Aibara, N., Ichinose, K., Baba, M., Nakajima, H., Satoh, K., Atarashi, R., Kishikawa, N., Nishida, N., Kawakami, A., Kuroda, N., Ohyama, K., 2018. Proteomic approach to profiling immune complex antigens in cerebrospinal fluid samples from patients with central nervous system autoimmune diseases. Clinica Chimica Acta 484, 26-31.
Alam, M.T., Nagao-Kitamoto, H., Ohga, N., Akiyama, K., Maishi, N., Kawamoto, T., Shinohara, N., Taketomi, A., Shindoh, M., Hida, Y., Hida, K., 2014. Suprabasin as a novel tumor endothelial cell marker. Cancer Sci 105, 1533-1540.
Blanpain, C., Mohrin, M., Sotiropoulou, P.A., Passegue, E., 2011. DNA-damage response in tissue-specific and cancer stem cells. Cell Stem Cell 8, 16-29.

Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
Burrell, R.A., McGranahan, N., Bartek, J., Swanton, C., 2013. The causes and consequences of genetic heterogeneity in cancer evolution. Nature 501, 338-345.

Cho, H.J., Kim, S.K., Jin, S.M., Hwang, E.M., Kim, Y.S., Huh, K., Mook-Jung, I., 2007. IFN-gamma-induced BACE1 expression is mediated by activation of JAK2 and ERK1/2 signaling pathways and direct binding of STAT1 to BACE1 promoter in astrocytes. Glia 55, 253-262.

Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26, 1367-1372.

Cox, J., Mann, M., 2012. 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. BMC Bioinformatics 13 Suppl 16, S12.

Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A 95, 15623-15628.

Deschênes-Simard, X., Parisotto, M., Rowell, M.-C., Le Calvé, B., Igelmann, S., Moineau-Vallée, K., Saint-Germain, E., Kalegari, P., Bourdeau, V., Kottakis, F., Bardeesy, N., Ferbeyre, G., 2018. Circumventing senescence is associated with stem cell properties and metformin sensitivity. Aging Cell doi:10.1111/acel.12889, e12889.

Dunning, M.J., Smith, M.L., Ritchie, M.E., Tavare, S., 2007. beadarray: R classes and methods for Illumina bead-based data. Bioinformatics 23, 2183-2184.

Di Vizio, D., Kim, J., Hager, M.H., Morello, M., Yang, W., Lafargue, C.J., True, L.D., Rubin, M.A., Adam, R.M., Beroukhim, R., Demichelis, F., Freeman, M.R., 2009. Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease. Cancer Res 69, 5601-5609.

Gaykalova, D., Vatapalli, R., Glazer, C.A., Bhan, S., Shao, C., Sidransky, D., Ha, P.K., Califano, J.A., 2012. Dose-dependent activation of putative oncogene SBSN by BORIS. PLoS One 7, e40389.

Ghisolfi, L., Keates, A.C., Hu, X., Lee, D.K., Li, C.J., 2012. Ionizing radiation induces stemness in cancer cells. PLoS One 7, e43628.

Glazer, C.A., Smith, I.M., Ochs, M.F., Begum, S., Westra, W., Chang, S.S., Sun, W., Bhan, S., Khan, Z., Ahrendt, S., Califano, J.A., 2009. Integrative discovery of epigenetically derepressed cancer testis antigens in NSCLC. PLoS One 4, e8189.

Gomez-Casal, R., Bhattacharya, C., Ganesh, N., Bailey, L., Basse, P., Gibson, M., Epperly, M., Levina, V., 2013. Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. Mol Cancer 12, 94.

Halfter, U.M., Derbyshire, Z.E., Vaillancourt, R.R., 2005. Interferon-gamma-dependent tyrosine phosphorylation of MEKK4 via Pyk2 is regulated by annexin II and SHP2 in keratinocytes. Biochem J 388, 17-28.

Han, X.Y., Wei, B., Fang, J.F., Zhang, S., Zhang, F.C., Zhang, H.B., Lan, T.Y., Lu, H.Q., Wei, H.B., 2013. Epithelial-mesenchymal transition associates with maintenance of stemness in spheroid-derived stem-like colon cancer cells. PLoS One 8, e73341.

Harris, J.E., Fernandez-Vilaseca, M., Elkington, P.T., Horncastle, D.E., Graeber, M.B., Friedland, J.S., 2007. IFNgamma synergizes with IL-1beta to up-regulate MMP-9 secretion in a cellular model of central nervous system tuberculosis. FASEB J 21, 356-365.
Hubackova, S., Kucerova, A., Michlits, G., Kyjacova, L., Reinis, M., Korolov, O., Bartek, J., Hodny, Z., 2015. IFN[gamma] induces oxidative stress, DNA damage and tumor cell senescence via TGF[beta]/SMAD signaling-dependent induction of Nox4 and suppression of ANT2. Oncogene 35, 1236-1249.

Kornblith, A.B., Herndon, J.E., 2nd, Silverman, L.R., Demakos, E.P., Odchimar-Reissig, R., Holland, J.F., Powell, B.L., DeCastro, C., Ellerton, J., Larson, R.A., Schiffer, C.A., Holland, J.C., 2002. Impact of azacytidine on the quality of life of patients with myelodysplastic syndrome treated in a randomized phase III trial: a Cancer and Leukemia Group B study. J Clin Oncol 20, 2441-2452.

Kreso, A., O’Brien, C.A., van Galen, P., Gan, O.I., Notta, F., Brown, A.M., Ng, K., Ma, J., Wienholds, E., Dunant, C., Pollett, A., Gallinger, S., McPherson, J., Mullighan, C.G., Shibata, D., Dick, J.E., 2013. Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. Science 339, 543-548.

Kyjacova, L., Hubackova, S., Krejcikova, K., Strauss, R., Hanzlikova, H., Dzijak, R., Imrichova, T., Simova, J., Reinis, M., Bartek, J., Hodny, Z., 2015. Radiotherapy-induced plasticity of prostate cancer mobilizes stem-like non-adherent, Erk signaling-dependent cells. Cell Death Differ 22, 898-911.

Levina, V., Marrangoni, A.M., DeMarco, R., Gorelik, E., Lokshin, A.E., 2008. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. PLoS One 3, e3077.

Medici, D., Hay, E.D., Olsen, B.R., 2008. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. Mol Biol Cell 19, 4875-4887.

Moncharmont, C., Levy, A., Guy, J.B., Falk, A.T., Guilbert, M., Trone, J.C., Alphonse, G., Gilormini, M., Ardail, D., Toillon, R.A., Rodriguez-Lafresse, C., Magne, N., 2014. Radiation-enhanced cell migration/invasion process: a review. Crit Rev Oncol Hematol 92, 133-142.

Nusse, R., 2008. Wnt signaling and stem cell control. Cell Res 18, 523-527.

Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., Mann, M., 2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1, 376-386.

Park, G.T., Lim, S.E., Jang, S.I., Morasso, M.I., 2002. Suprabasin, a novel epidermal differentiation marker and potential cornified envelope precursor. J Biol Chem 277, 45195-45202.

Pulaski, B.A., Ostrand-Rosenberg, S., 2001. Mouse 4T1 breast tumor model. Current protocols in immunology / edited by John E. Coligan ... [et al.] Chapter 20, Unit 20.22.

Rak, J., 2010. Microparticles in cancer. Semin Thromb Hemost 36, 888-906.

Rhim, A.D., Mirek, E.T., Aiello, N.M., Maitra, A., Bailey, J.M., McAllister, F., Reichert, M., Beatty, G.L., Rustgi, A.K., Vonderheide, R.H., Leach, S.D., Stanger, B.Z., 2012. EMT and Dissemination Precede Pancreatic Tumor Formation. Cell 148, 349-361.
Rudek, M.A., Zhao, M., He, P., Hartke, C., Gilbert, J., Gore, S.D., Carducci, M.A., Baker, S.D., 2005. Pharmacokinetics of 5-azacitidine administered with phenylbutyrate in patients with refractory solid tumors or hematologic malignancies. J Clin Oncol 23, 3906-3911.

Ruegg, C., Monnier, Y., Kuonen, F., Imaizumi, N., 2011. Radiation-induced modifications of the tumor microenvironment promote metastasis. Bull Cancer 98, 47-57.

Sakatsume, M., Stancato, L.F., David, M., Silvennoinen, O., Saharinen, P., Pierce, J., Larner, A.C., Finbloom, D.S., 1998. Interferongamma Activation of Raf-1 Is Jak1-dependent and p21ras-independent. J. Biol. Chem. 273, 3021-3026.

Sanchez-Tillo, E., de Barrios, O., Siles, L., Cuatrecasas, M., Castells, A., Postigo, A., 2011. beta-catenin/TCF4 complex induces the epithelial-to-mesenchymal transition (EMT)-activator ZEB1 to regulate tumor invasiveness. Proc Natl Acad Sci U S A 108, 19204-19209.

Shao, C., Tan, M., Bishop, J.A., Liu, J., Bai, W., Gaykalova, D.A., Ogawa, T., Vikani, A.R., Agrawal, Y., Li, R.J., Kim, M.S., Westra, W.H., Sidransky, D., Califano, J.A., Ha, P.K., 2012. Suprabasin is hypomethylated and associated with metastasis in salivary adenoid cystic carcinoma. PLoS One 7, e48582.

Sheng, S.H., Zhu, H.L., 2014. Proteomic analysis of pleural effusion from lung adenocarcinoma patients by shotgun strategy. Clin Transl Oncol 16, 153-157.

Skvortsova, I., Skvortsov, S., Stasyk, T., Raju, U., Popper, B.A., Schiestl, B., von Guggenberg, E., Neher, A., Bonn, G.K., Huber, L.A., Lukas, P., 2008. Intracellular signaling pathways regulating radioresistance of human prostate carcinoma cells. Proteomics 8, 4521-4533.

Smyth, G.K., Michaud, J., Scott, H.S., 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21, 2067-2075.

Stancato, L.F., Yu, C.R., Petricoin, E.F., 3rd, Larner, A.C., 1998. Activation of Raf-1 by interferon gamma and oncostatin M requires expression of the Stat1 transcription factor. J Biol Chem 273, 18701-18704.

Sun, L., Wu, J., Du, F., Chen, X., Chen, Z.J., 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339, 786-791.

Swanton, C., 2012. Intratumor heterogeneity: evolution through space and time. Cancer Res 72, 4875-4882.

Tarca, A.L., Draghici, S., Khatri, P., Hassan, S.S., Mittal, P., Kim, J.S., Kim, C.J., Kusanovic, J.P., Romero, R., 2009. A novel signaling pathway impact analysis. Bioinformatics 25, 75-82.

Thiery, J.P., Acloque, H., Huang, R.Y., Nieto, M.A., 2009. Epithelial-mesenchymal transitions in development and disease. Cell 139, 871-890.

Tyanova, S., Temu, T., Sinticyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., Cox, J., 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods 13, 731-740.

van Neerven, S.M., Tieken, M., Vermeulen, L., Bijlsma, M.F., 2016. Bidirectional interconversion of stem and non-stem cancer cell populations: A reassessment of theoretical models for tumor heterogeneity. Molecular & cellular oncology 3, e1098791.

Vlasakova, J., Novakova, Z., Rossmeislova, L., Kahle, M., Hozak, P., Hodny, Z., 2007. Histone deacetylase inhibitors suppress IFN[alpha]-induced up-regulation of promyelocytic leukemia protein. Blood 109, 1373-1380.

Zhu, J., Wu, G., Li, Q., Gong, H., Song, J., Cao, L., Wu, S., Song, L., Jiang, L., 2016. Overexpression of Suprabasin is Associated with Proliferation and Tumorigenicity of Esophageal Squamous Cell Carcinoma. Scientific reports 6, 21549.
Legends and Figures

Figure 1. (a) Schematic representation of the treatment protocol using either multiple doses of 5-AC every 24 hours (left) or a single dose of 5-AC (right) resulting in generation of chemoresistant populations (adherent, low-adherent and re-adherent) in human HeLa and MCF-7 cancer cell lines or HS-5 cells. (b) Loss of adhesion expressed as a ratio (fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells (see Material and Methods) assessed by FACS in control cells or cells treated with 2 µM 5-AC for 7 days. (c) RT-qPCR quantification of Sox2 and CD44 mRNA levels in parental, adherent and low-adherent HeLa cells treated with 4 µM 5-AC for 7 days. (d) RT-qPCR quantification of DLL1, DLL4, Hes1 and Hey1 mRNA levels in parental, adherent and low-adherent HeLa cells treated with 4 µM 5-AC for 7 days. (e) Immunoblotting detection of Snail in parental, adherent and low-adherent HeLa and MCF-7 cells treated with 4 µM 5-AC for 72 hours. GAPDH was used as a loading control. (f) RT-qPCR quantification of p16 and p21 mRNA levels in parental, adherent and low-adherent HeLa cells treated with 2 µM 5-AC for 72 hours. (g) Immunoblotting detection of Bcl-XL in parental, adherent and low-adherent HeLa cells treated with 4 µM 5-AC for 72 hours. γ-tubulin was used as a loading control. (h) Estimation of low-adherent fraction after inhibition of Erk kinase pathway with selumetinib (ERKi, 1 µM) in combination with siRNA knockdown of Bim (siBim) expressed as a ratio (in fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in HeLa cells treated with 2 µM 5-AC for 72 hours. Non-targeting siRNA (siNC) was used as a control. (i) Immunoblotting detection of Erk phosphorylated on threonine 202/tyrosine 204 and Bim in HeLa cells treated with 2 µM 5-AC for 72 hours after knockdown of Bim (siBim) or in the presence of MEK/Erk inhibitor selumetinib (ERKi; 1 µM). GAPDH was used as a loading control. Data are shown as mean values ± SEM, with n = 3 per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey’s post hoc test. The asterisk represents p < 0.05.

Figure 2. (a) Heat map showing the hierarchical clustering of LFC of genes commonly deregulated in all three low-adherent cell fractions after exposure to stress (DU145 and MCF-7, 10 × 2 Gy; HeLa, 7 × 4 µM 5-AC). (b) Heat map showing expression of interferon-regulated genes selected from cluster I in all three low-adherent cell fractions after exposure to stress (DU145 and MCF-7, 10 × 2 Gy; HeLa, 7 × 4 µM 5-AC). (c) RT-qPCR validation of Molecular Oncology (2019) © 2019 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.
changes in the expression of selected genes from microarray analysis in 5-AC (4 μM)-treated HeLa cells. Data are shown as mean values ± SEM, with \( n = 3 \) per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey's *post hoc* test. The asterisk represents \( p < 0.05 \).

**Figure 3.** (a) Loss of adhesion expressed as ratio (in fold induction) of AnnexinV−/Hoechst−detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in HeLa and MCF-7 cells treated with IFN\( \gamma \) (5 ng/ml) or IFN\( \beta \) (50 ng/ml) for 3 days (HeLa) or 6 days (MCF7). Non-treated cells were used as a control. Immunoblotting detection of STAT1 phosphorylated on tyrosine 701, IRF1 and IRF7 in control and IFN\( \gamma \)- or IFN\( \beta \)-treated HeLa (b) or MCF-7 (c) cells for 3 or 6 days. GAPDH was used as a loading control. (d) Correlation of mRNA and protein expression of selected interferon targets in irradiated (10 × 2 Gy) DU145 non-adherent cells compared to control cells based on annotations.

Figure 3. (a) Loss of adhesion expressed as ratio (in fold induction) of AnnexinV−/Hoechst−detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in HeLa and MCF-7 cells treated with IFN\( \gamma \) (5 ng/ml) or IFN\( \beta \) (50 ng/ml) for 3 days (HeLa) or 6 days (MCF7). Non-treated cells were used as a control. Immunoblotting detection of STAT1 phosphorylated on tyrosine 701, IRF1 and IRF7 in control and IFN\( \gamma \)- or IFN\( \beta \)-treated HeLa (b) or MCF-7 (c) cells for 3 or 6 days. GAPDH was used as a loading control. (d) RT-qPCR quantification of Sox2 and Oct4 mRNA levels in parental, adherent and low-adherent HeLa cells treated with IFN\( \gamma \) for 72 hours. (e) Immunoblotting detection of STAT1 phosphorylated on tyrosine 701 and Erk phosphorylated on threonine 202/tyrosine 204 in HeLa cells treated with IFN\( \gamma \) for the period as indicated. GAPDH was used as a loading control. (f) Immunoblotting detection of STAT1 phosphorylated on tyrosine 701 and Erk phosphorylated on threonine 202/tyrosine 204 in MCF-7 cells treated with IFN\( \gamma \) for 72 hours. GAPDH was used as a loading control. (g) Estimation of low-adherent survival after inhibition of Erk kinase with selumetinib (ERKi; 1 μM) expressed as indicated (see above) in HeLa cells treated with IFN\( \gamma \) for 72 hours. (h) Clonogenic cell survival assay of HeLa cells treated with IFN\( \gamma \) alone or in combination with Erk inhibition with selumetinib (ERKi; 1 μM) for 72 hours and assayed at day 29 after the end of the treatment. (i) Effect of siRNA knockdown of IRF1 (siIRF1) on the loss of adhesion expressed as indicated (see above) in HeLa and MCF-7 cells treated with IFN\( \gamma \) for 72 hours. Non-targeting siRNA (siNC) was used as a control. (j) Effect of siRNA knockdown of IRF1 (siIRF1) on the loss of adhesion expressed as indicated (see above) in DU145 and MCF-7 cells irradiated with one dose of 10 Gy for 72 hours. Non-targeting siRNA (siNC) was used as a control. Data are shown as mean values ± SEM, with \( n = 3 \) per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey's *post hoc* test. The asterisk represents \( p < 0.05 \).
Figure 4. RT-qPCR quantification of SBSN mRNA level in parental, adherent and low-adherent HeLa cells treated for 7 days with 4 μM 5-AC (a), DU145 cells irradiated (fIR) daily with 2 Gy for 10 days (b), and HeLa cells treated with IFNγ (5 ng/ml) for 72 hours (c). Immunoblotting detection of SBSN in parental, adherent and low-adherent HeLa cells treated with 4 μM 5-AC for 5 days (d), DU145 cells irradiated (fIR) daily with 2 Gy for 10 days (e) and HeLa cells treated with IFNγ for 72 hours (f). GAPDH and ACTN1 were used as loading controls. (g) Effect of siRNA knockdown of SBSN (siSBSN) on the loss of adhesion expressed as ratio (in fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in HeLa cells treated with IFNγ for 72 hours. Non-targeting siRNA (siNC) was used as a control. (h) Effect of siRNA knockdown of SBSN (siSBSN) on the loss of adhesion expressed as ratio (in fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in HeLa and MCF-7 cells treated with 2 μM 5-AC for 72 hours. Non-targeting siRNA (siNC) was used as a control. (i) FACS analysis of the effect of SBSN siRNA-mediated (siSBSN) down regulation in U373 cells on the mobilization of low adhesion cell subpopulation after 2 Gy irradiation. The amount of low-adherent cells is expressed as a ratio (fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells. Non-targeting siRNA (siNC) was used as a control. Data are shown as mean values ± SEM, with n = 3 per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey's post hoc test. The asterisk represents p < 0.05.

Figure 5. (a) Effect of siRNA knockdown of IRF1 (siIRF1) on the expression of SBSN gene detected by RT-qPCR in HeLa cells treated with IFNγ (5 ng/ml) for 72 hours. Non-targeting siRNA (siNC) was used as a control. (b) Effect of siRNA knockdown of IRF1 (siIRF1) on Erk phosphorylated on threonine 202/tyrosine 204 detected by immunoblotting in HeLa cells treated with IFNγ for 72 hours. GAPDH was used as a loading control. Effect of Erk kinase inhibition by selumetinib (ERKi; 1 μM) on the SBSN expression assessed by RT-qPCR in HeLa cells treated with IFNγ (5 ng/ml) (e) or 2 μM 5-AC (d) for 72 hours. Effect of siRNA knockdown of Erk1 (siErk1) or Erk2 (siErk2) on the SBSN expression assessed by RT-qPCR in HeLa cells treated with IFNγ (e) or 2 μM 5-AC (f) for 72 hours. Non-targeting siRNA (siNC) was used as a control. (g) SBSN proximal promoter-driven Firefly luciferase activity in HEK293 cells exposed to IFNγ (5 ng/ml), MEK/Erk inhibitor selumetinib (ERKi; 1 μM), their combination and PMA (20 nM) for 48 hours. SBSN luciferase activity for each
condition is expressed relatively to promoterless pGL3-Basic construct. Data are shown as mean values ± SEM, with n = 3 per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey's post hoc test. The asterisk represents p < 0.05.

**Figure 6.** (a) RT-qPCR quantification of SBSN mRNA level in human colorectal tumours. Two samples from two different tumour sites were analysed (T1, T2; for statistic evaluation, values from T1 and T2 were averaged) and compared to patients healthy surrounding mucosa (HM), with n = 8 per group. (b) RT-qPCR quantification of SBSN mRNA level in human ovarian cancer samples, with n = 14 for healthy controls and n = 100 for ovarian carcinoma samples. RT-qPCR quantification of SBSN (c) and Sox2 (d) mRNA level in 4T1 parental cells (grown in vitro), 4T1 cells forming solid tumour, 4T1 cells circulating in blood (CTC) and 4T1 metastasis in lungs and liver of Balb/c mice, with n = 5 to 6 per group. Data are shown as mean values ± SEM. Two-way ANOVA was used for multigroup comparisons followed by Tukey's post hoc test. The asterisk represents p < 0.05.

**Supporting Information**

**Supplementary Figure 1**

**Fig. S1.** Generation of the low-adherent stem cell-like cells with 5-AC. (a) Induction of apoptosis expressed as a percentage of AnnexinV+/Hoechst+ cells assessed by FACS in control or HeLa cells treated with 5-AC (2 µM) for 72 hours. (b) Immunofluorescence detection of DAPI (blue) and autofluorescent signal (green) in low-adherent HeLa cells treated with 2 µM 5-AC for 72 hours. Scale bar 10 µm. (c) Phase contrast microscopic images of chemoresistant MCF-7 cell populations 16 and 24 days after treatment with 0.5 µM 5-AC. Cells were treated for 7 days, low-adherent cells were then removed and cultivated in fresh medium. Scale bar 100 µm. RT-qPCR quantification of Sox2 (d), Hes1, DLL1, DLL4 (f) and p21 and p16 (i) expression in parental, adherent and low-adherent MCF-7 cells treated with 2 µM 5-AC for 72 hours. RT-qPCR quantification of Sox2 and OCT4 (e), Hes1, Hey1, DLL4 (g) and p21 and p16 (j) expression in parental, adherent and low-adherent HS-5 cells treated with 2 µM 5-AC for 72 hours. (h) RT-qPCR quantification of Snail expression in parental, adherent and low-adherent MCF-7, HeLa and HS-5 cells treated with 2 µM 5-AC for 72 hours. (k) Immunoblotting detection of E-cadherin
and ITGAV in parental, adherent and low-adherent MCF-7 cells treated with 4 μM 5-AC for 72 hours. GAPDH was used as a loading control. (l) Immunoblotting detection of total and threonine 202/tyrosine 204-phosphorylated Erk and total and serine 473-phosphorylated Akt in HeLa and MCF-7 cells treated with 4 μM 5-AC for 24 and 48 hours. GAPDH was used as a loading control. (m) Clonogenic cell survival assay of HeLa cells treated with 2 μM 5-AC for 72 hours in the presence of MEK/Erk inhibitor selumetinib (ERKi; 1 μM). Surviving readherent HeLa cells were detected on day 24 following treatment. Data are shown as mean values ± SEM, with n = 3 per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey's post hoc test. The asterisk represents p < 0.05.

**Supplementary Figure 2**

**Fig. S2.** Analysis of proteome and transcriptome of 5-AC- and fIR-treated cells.

(a) Genes in GOBP category: “response to type I interferon” with their logarithm fold change values compared to control. (b) Correlation of mRNA and protein expression in irradiated (10 × 2 Gy) DU145 low-adherent cells compared to control cells based on annotations. Numbers on the X or Y axis represent the score achieved in 2D annotation enrichment analysis. Terms with both coordinates positive and negative are upregulated/downregulated concomitantly in the proteome and transcriptome, the rest is regulated discordantly. (c) Table containing the exact terms that have both score values larger than 0.4 or smaller than -0.4.

**Supplementary Figure 3**

**Fig. S3.** IFNγ induced the low-adherent phenotype.

(a) Loss of adhesion expressed as a ratio (fold induction) of AnnexinV−/Hoechst− detached cells relative to all AnnexinV−/Hoechst− cells assessed by FACS in control HS-5 cells or cells treated with IFNγ (5 ng/ml) for 3 days. (b) Phase contrast microscopic image of MCF-7 cells irradiated for 6 days with one dose of 10 Gy. Large extracellular vesicles are marked with yellow arrows. Scale bar 10 μm. (c) Immunoblotting detection of STAT1 phosphorylated on tyrosine 701 and Erk1/2 phosphorylated on threonine 202/tyrosine 204 in HeLa cells treated with IFNγ (5 ng/ml) for 72 hours in the presence of MEK/Erk inhibitor selumetinib (ERKi; 1 μM). GAPDH was used as a loading control. (d) Immunoblotting detection of STAT1 phosphorylated on tyrosine 701 and IRF1 in HeLa and MCF-7 cells treated with IFNγ (5 ng/ml) for 72 hours after knockdown of IRF1 (siIRF1). Non-targeting siRNA (siNC) was used as a control. GAPDH was used as a loading control. (e)
Immunoblotting detection of IRF1 in DU145 and MCF-7 cells 72 hours after irradiation with one dose of 10 Gy after knockdown of IRF1 (siIRF1). Non-targeting siRNA (siNC) was used as a control. GAPDH was used as a loading control. Data are shown as mean values ± SEM, with n = 3. Two – tailed unpaired Student's t-test was used for comparison between two groups for statistical significance. The asterisk represents p < 0.05.

**Supplementary Figure 4**

**Fig. S4.** Transcriptome analysis of cell populations after fIR and 5-AC exposure and SBSN detection.

(a) Venn diagram of upregulated genes in low-adherent HeLa cells treated with 4 μM 5-AC for 7 days and MCF-7 and DU145 irradiated daily with 2 Gy for 10 days. Numbers represent single and common significantly up- or down-regulated genes in the control (non-treated) sample. (b) Heat map showing the hierarchical clustering of LFC of genes commonly upregulated in all three low-adherent cell fractions after exposure to stress (DU145, 10 × 2 Gy; MCF-7, 10 × 2 Gy, HeLa, 5-AC). (c) RT-qPCR quantification of SBSN expression in HeLa cells after SBSN knockdown (siSBSN) treated with IFNγ (5 ng/ml) for 72 hours. (d) RT-qPCR quantification of SBSN expression in HeLa and MCF-7 cells with SBSN knock down (siSBSN) treated with 4 μM 5-AC for 72 hours. Immunoblotting detection of the SBSN isoforms in the cell lysates (e) and conditioned media (f) of the U373 and SK-OV-3 cells. The SBSN signal was suppressed by the SBSN siRNA (siSBSN; 48 hours after the RNAi-mediated knockdown of the SBSN). Non-targeting siRNA (siNC) was used as a control. Ponceau S staining was used for a control of protein loading. Immunofluorescence detection of the SBSN in the U373 cells irradiated with a single dose of 2 Gy using LS-C162878 (g) or HPA067734 (h) antibodies. Nuclei were stained with DAPI (1 μg/ml). Scale bar 10 μm. (i) Quantitative FACS analysis of apoptosis using AnnexinV/Hoechst staining of non-irradiated (control) or single dose (2 Gy)-irradiated U373 cells. Non-targeting siRNA (siNC) was used as a control. Data are shown as mean values ± SEM, with n = 3. Two-way ANOVA was used for multigroup comparisons followed by Tukey's post hoc test. The asterisk represents p < 0.05.
Supplementary Figure 5

Fig. S5. Effect of the ERK1/2 pathway on the survival of cells exposed to 5-AC.

(a) Immunoblotting detection of total Erk1/2 in HeLa cells treated with IFNγ (5 ng/ml) for 72 hours after knockdown of Erk1 (siErk1) or Erk2 (siErk2). Non-targeting siRNA (siNC) was used as a control. GAPDH was used as a loading control. (b) Immunoblotting detection of Erk phosphorylated on threonine 202/tyrosine 204 in HeLa cells treated with 2 μM 5-AC for 72 hours in the presence of MEK/Erk inhibitor selumetinib (ERKi; 1 μM). GAPDH was used as a loading control. (c) RT-qPCR quantification of SBSN expression in MCF-7 cells treated with 2 μM 5-AC for 72 hours in the presence of MEK/Erk inhibitors selumetinib (ERKi; 1 μM) or U0126 (10 μM). (d) Immunoblotting detection of Erk phosphorylated on threonine 202/tyrosine 204 in MCF-7 cells treated with 2 μM 5-AC for 72 hours in the presence of MEK/Erk inhibitors selumetinib (ERKi; 1 μM) or U0126 (10 μM). GAPDH was used as a loading control. (e) Immunoblotting detection of Erk1 and Erk2 in HeLa cells treated with 2 μM 5-AC for 72 hours after knockdown of Erk1 (siErk1) or Erk2 (siErk2). Non-targeting siRNA (siNC) was used as a control. GAPDH was used as a loading control. Data are shown as mean values ± SEM, with n = 3 per group. Two-way ANOVA was used for multigroup comparisons followed by Tukey’s post hoc test.

Supplemental Tables

Supplemental Table 1

Table S1. Transcriptome analysis of cells surviving fIR and 5-AC treatment.

Excel table containing the log2 fold-change (log2FC) of mRNA expression significantly deregulated in irradiated (10 × 2 Gy) DU145 and MCF-7 cells and 5-AC (7 × 4 μM)-treated HeLa cells compared to non-irradiated, non-treated control cells (1a) and results from annotation enrichment performed on clusters from the heat map (1b). P – values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method.

Supplemental Table 2

Table S2. Changes of functional categories on the proteome and transcriptome level of irradiated low-adherent DU145 cells.

Gene ontology (GO) biological processes (GOBP), molecular functions (GOMF), cellular compartments (GOCC), and KEGG pathways were analyzed with 1D protein (2a) and 2D protein and mRNA annotation enrichment (2b) obtained from the comparison of irradiated...
(10 × 2 Gy) low-adherent and non-irradiated control DU145 cells. P – values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) method.

Supplemental Table 3

Table S3. Description of human colon carcinoma and ovarian cancer samples.

Video S1

Video of the amoeboid-like form of cell migration of low-adherent cells.
**Protein names**

| Protein names | Gene names | LogFC protein | LogFC RNA | p-value protein | p-value RNA |
|---------------|------------|----------------|-----------|-----------------|-------------|
| HLA class I histocompatibility antigen, Cw-7 alpha chain | HLA-C | 3.483 | 0.356 | 3.8E-07 | 5.8E-03 |
| Non-receptor tyrosine-protein kinase PTK2 | TYK2 | 1.715 | 0.480 | 3.1E-22 | 3.1E-07 |
| Interface-induced transmembrane protein 3:2:1 | IFITM1-3 | 1.492 | 0.395 | 2.0E-05 | 2.0E-05 |
| 2,5-diketo-3[1H]pyrrolin-4-one synthase 1 | OAS1 | 3.197 | 0.030 | 9.0E-10 | 9.0E-09 |
| Interface-induced protein with tetrascariopod repeats 3 | FIT3 | 1.368 | 1.549 | 3.0E-09 | 3.0E-08 |
| Muscular-like protein S9G15 | S9G15 | 3.742 | 1.450 | 1.7E-16 | 1.7E-16 |
| Interface-induced protein with tetrascariopod repeats 2 | FIT2 | 1.929 | 0.420 | 3.3E-01 | 3.3E-01 |
| Interface-induced protein with tetrascariopod repeats 1 | FIT1 | 1.182 | 0.378 | 3.0E-04 | 3.0E-04 |
| HLA class I histocompatibility antigen, alpha chain E | HLA-E | 3.765 | 0.839 | 4.2E-03 | 4.2E-03 |
| HLA class I histocompatibility antigen, alpha chain G | HLA-G | 3.845 | 0.720 | 4.0E-09 | 4.0E-09 |
| Tyrosine-protein phosphatase non-receptor type 1 | PPP1R1B | 3.656 | 0.800 | 4.0E-10 | 4.0E-10 |
| Interface-induced GTP binding protein Mr1 | MR1 | 2.995 | 0.235 | 7.0E-18 | 7.0E-18 |
| Tyrosine-protein kinase JAK1 | JAK1 | 1.508 | 0.556 | 9.0E-45 | 9.0E-45 |
| Tyrosine-protein phosphatase non-receptor type 8 | PPP1R8 | 3.493 | 0.040 | 9.1E-14 | 9.1E-14 |
| Signal transducer and activator of transcription 1-alpha/beta | STAT1 | 3.241 | 0.586 | 9.0E-12 | 9.0E-12 |
| Signal transducer and activator of transcription 2 | STAT2 | 0.515 | 0.220 | 1.0E-14 | 1.0E-14 |
| Tyrosine-protein phosphatase non-receptor type 11 | PPP1R11 | 0.139 | 0.080 | 1.1E-01 | 1.1E-01 |
| Interface regulatory factor 3 | IFR3 | 0.479 | 0.110 | 2.5E-23 | 2.5E-23 |
| Zn2+ and calcium-dependent protein kinase | ZKSCAN5 | 1.338 | 1.380 | 3.6E-04 | 3.6E-04 |
| Interface-stimulated gene 20 kDa protein | ISG20 | 3.998 | 1.989 | 4.0E-14 | 4.0E-14 |
| Ubi fusion terminal hydrolase 1 | UFT1 | 3.279 | 0.298 | 3.4E-17 | 3.4E-17 |
| Zn2+ and calcium-dependent protein kinase | ZKSCAN5 | 1.059 | 0.136 | 1.1E-22 | 1.1E-22 |
