Delineating the DNA damage response using systems biology approaches

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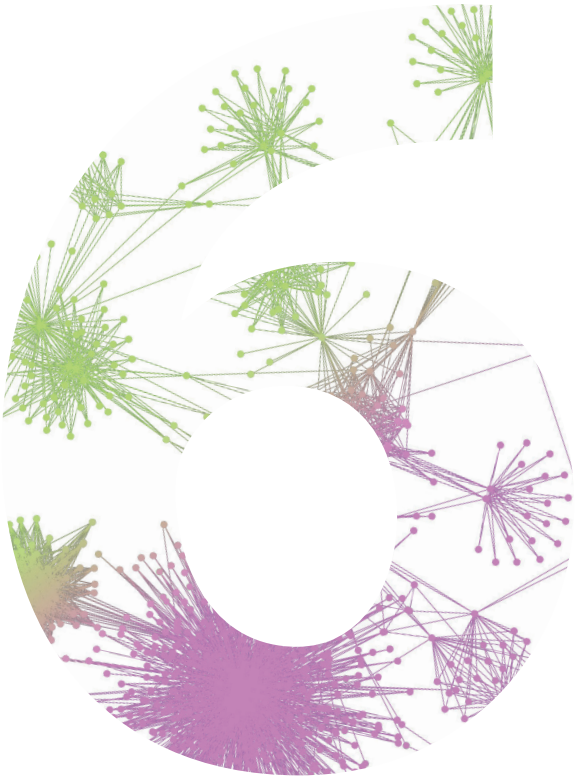
RNAI SCREEN FOR TARGETS FOR CHEMO-SENSITIZATION IDENTIFIES THE DUAL-SPECIFIC PHOSPHATASE DUSP15 AS A COMMON REGULATOR OF DISTINCT PRO-TECTIVE PATHWAYS IN VARIOUS CANCER CELL TYPES



MANUSCRIPT IN PREPARATION

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ABSTRACT

DNA damage-inducing cancer therapy is common treatment for various tumor types. However, acquired or native resistance hampers the success of chemo- or radiotherapy. To maintain survival in the presence of severe DNA damage, cancer cells often abrogate activity of the p53 tumor suppressor or corrupt mechanisms of apoptosis. Here, we have performed RNAi screens to detect novel targets for sensitization in cancer cells of varying genetic backgrounds, including those lacking a functional p53- or caspase 3-mediated response to DNA damage. A set of 62 siRNAs derived from a previous kinase/ phosphatase/ ubiquitinase/ transcription factor screen in ES cells that effectively sensitized ES cells to cisplatin, were analyzed in a panel of cancer cell lines. Four siRNAs were identified that sensitized all tested cancer cell lines. These targeted the protein kinase D subunit PRKCM, the E3 ubiquitin ligase ARIH1, the ribosomal protein Rpl7l1, and the dual-specific phosphatase DUSP15. DUSP15 has not been previously connected to the DNA damage response. In p53- and caspase-3 wild type cancer cells that show cisplatin-induced apoptosis, p38 MAPK activity was cisplatin-responsive and DUSP15 silencing caused enhanced p38 activity as determined by phosphorylation of the p38 pathway target Hsp27. In these cells, pharmacological inhibition of p38 activity blocked DUSP15-mediated sensitization. Conversely, p53/caspase-3-deficient cells showed no cisplatin-regulated p38 activity and the observed DUSP15-mediated sensitization in those cells was not affected by p38 inhibition. These findings identify DUSP15 as a common target for cancer cell chemosensitization that can act through distinct, p38-dependent or independent mechanisms.

INTRODUCTION

Chemo- and radio-cancer therapy often relies on DNA damage-induced killing of tumor cells. Typically, tumor cells have features that make them more susceptible to genotoxic stress, such as a generally high proliferation rate, as well as defective DNA damage repair and checkpoint signaling [1; 2; 3]. However, native or acquired resistance frequently hampers successful patient cure [1]. Many recent studies have aimed at identifying new drug targets to improve the efficacy of cancer therapy, preferably by exploiting cancer cell inherent deficiencies in DNA damage checkpoints and DNA repair [2].

Genotoxic stress can induce various cellular responses, including a halt of the cell cycle, DNA repair, but also induction of cell killing by apoptosis or senescence [4; 5]. The outcome of DNA damage induction strongly depends on the cellular background. Often the functionality of a few key DNA damage response (DDR) signaling molecules is crucial in determining cellular decision making in the presence of DNA damage. The transcription factor p53 that has been termed the "guardian of the genome" plays a crucial role in orchestrating the DNA damage response and has been suggested to be mutated or its function otherwise blocked in more than half of all human cancers [6; 7]. p53 is vital in executing many DNA damage-induced programs, including DNA repair, cell cycle arrest and apoptosis [7]. The central role that p53 takes in executing DNA damage-induced functions, entails that the cellular response to DNA damage differs strongly between p53-proficient and deficient cells. Indeed, lack of p53 expression has in many cases been linked to resistance of tumor cells towards DNA damage-inducing therapy. Furthermore, p53 mutations, which allow its expression, but change its functionality can lead to the p53 protein acquiring tumor progression-promoting properties [8].

Cell death by apoptosis is a well described route of cell killing induced by DNA damage [9; 10]. However, also other forms of cell death such as autophagic cell death, necrosis, senescence and mitotic catastrophe can be important in cancer cell killing after DNA damage and can possibly be exploited for therapy responses [11].

Finding therapeutic targets, whose silencing can kill cancer cells irrespective of classical p53-mediated apoptosis is a major challenge on the way to improve the effectiveness of cancer therapy. Interestingly, a number of recent reports indicated the potential to exploit the deficiency of tumor cells in p53 controlled DNA damage responses. Absence of p53 function makes tumor cells vulnerable to checkpoint inhibition (e.g. by inhibition of ATM or MK2), leading them to undergo mitotic catastrophe [12; 13; 14].

We have recently identified genes whose silencing leads to enhanced sensitivity to the genotoxic drug cisplatin in mouse embryonic stem (ES) cells that readily undergo apoptosis [15]. In contrast to many cultured cell lines, ES cells do not require silencing of key tumor suppressor pathways (e.g. p53, p16, hTERT) in order to maintain growth in culture condition and therefore serve as a good model to study an intact DDR [15; 16]. Interestingly, ES cells have certain characteristics that can be extrapolated to cancer cells and particularly cancer stem cells such as lack of a G1/S-checkpoint and high proliferation rate [17; 18].

Identified ES cell targets, were silenced in a panel of cancer cells of varying genetic background with respect to p53 and caspase-3 status that showed different levels of increased cisplatin resistance as compared to ES cells. This led to the identification of a small set of genes whose silencing preferentially or exclusively reduced cell survival in the presence of cisplatin. One of these targets, the dual specific phosphatase, DUSP15 had not been previously implicated in the DDR and its silencing caused synthetic lethality in all lines. Interestingly, our data indicate that although identified as a common sensitizer, DUSP15 acts trough distinct, p38MAPK-mediated or independent pathways in different cancer cell types.

RESULTS

MCF7 and H1299 cells are resistant to cisplatin and show lack of p53-mediated apoptosis

In previous studies we have carried out siRNA SMARTpool screens for all cellular kinases and phosphatases, as well (de-) ubiquitinases, (de-) sumoylases and transcription factors. This led to the identification of 236 cisplatin response modulators. Deconvolution screening confirmed 62 genes, whose knockdown sensitized mouse ES cells to cisplatin-induced killing [15] (von Stechow et al., submitted) (Fig 1A). Pathway analysis implicated those genes in DNA damage repair, cell cycle checkpoint regulation, and other cancer-relevant pathways (Fig S1). Mouse ES cells are sensitive to cisplatin, showing ~60% cell death after treatment with 10 μM cisplatin for 24 h (Fig 1B; Fig S2A). In order to assess if these RNAi screen hits were similarly implicated in regulating survival of more resistant (cancer) cells we analyzed cisplatin sensitivity of a number of cancer cell lines. These included the mouse breast cancer cell line 4T1, the human breast cancer cell line MCF7, the human non-small-cell lung cancer (NSCLC) cell line H1299 and the human liver cancer cell line HepG2. Cisplatin sensitivities varied but all cancer cells were considerably more resistant than ES cells (Fig 1B; Fig S2B-E). For the siRNA screens, MCF7 (reported to be caspase-3-deficient; [19; 20]) and H1299 (reported to be p53-deficient; [21] were used.

We analyzed DNA damage-induced p53 accumulation and dependency of cisplatin-induced cell killing on p53 and caspase pathways in these cells. MCF7 cells showed an accumulation of active p53 after cisplatin treatment whereas, in agreement with earlier studies, no p53 was detected in H1299 NSCLC cells [21] (Fig 2A,B). Knockdown of p53 strongly protected ES cells against cisplatin-induced killing, led to a small but not significant protection in MCF7, but, in agreement with the absence of p53 protein levels, did not affect cisplatin-mediated killing of H1299 (Fig 2C-E). The minor effect of p53 knockdown in MCF7 cells, despite clear accumulation of active p53, indicates that alternative compensating pathways causing cell death must be activated by cisplatin in this cell type. To test the dependency of cell killing on caspase-mediated apoptosis

we co-treated cells with cisplatin and the pan-caspase inhibitor, ZVAD-fmk (ZVAD). While survival of ES cells in presence of cisplatin was rescued by ZVAD treatment, this effect was not observed in MCF7 or H1299 cells (Fig 2F-H). In agreement with lack of caspase-3-mediated apoptosis in both cell lines, appearance of an apoptotic subG1/G0-fraction was negligible in MCF7 and H1299 cells even after 48 h of cisplatin treatment (Fig S3A, B).

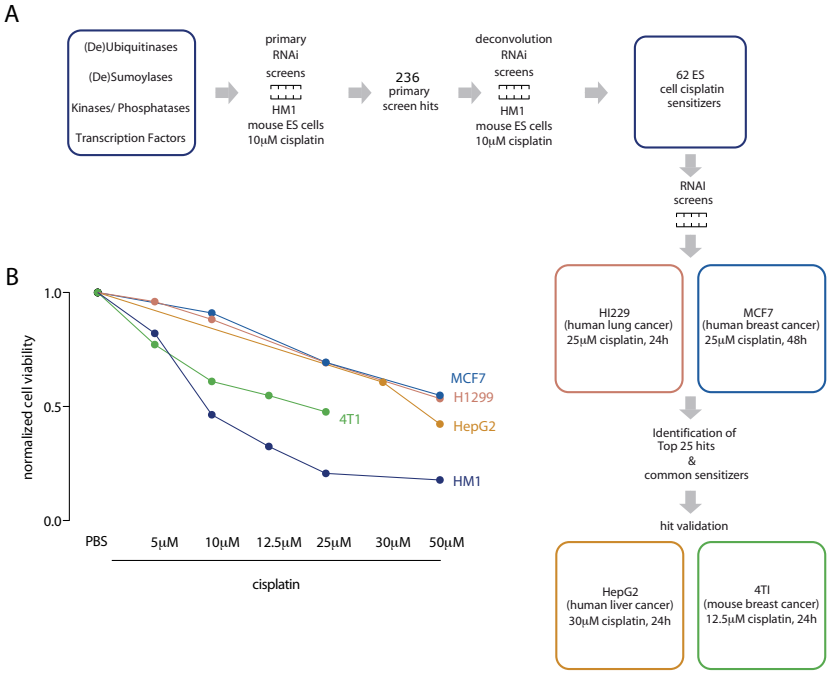

Figure 1. Identification of cisplatin resistant cancer cell lines. (A) scheme for RNAi screen experiments: in HM1 mouse ES cells RNAi screens led to identification of 62 validated cisplatin sensitizers. Hits were incorporated into H1299 and MCF7 cancer cells and further validated in 4T1 and HepG2 cells. (B) Normalized cell survival in HM1 mouse ES cells, 4T1 mouse breast cancer cells, HepG2 human liver cancer cells, H1299 human NSCLC cells and MCF7 human breast cancer cells treated for 24 h with the indicated cisplatin concentrations.

Selection of siRNAs that sensitize two cisplatin-resistant cancer cell lines

Next, we performed siRNA screens in H1299 and MCF7 to identify those hits from the ES cell screen that were confirmed as targets for sensitization. Cisplatin concentrations were chosen such that cell killing was ~25% using 25 μM for both lines and 24 h treatment for H1299 and 48 h treatment for MCF7 (Fig 1A,B; FigS 2B, C). Screening conditions were optimized using knockdown of Kif11 and GAPDH as transfection efficiency and knockdown controls, respectively. Two individual negative controls were used in the screening plates (Fig S4A, B).

siRNA screens were performed in duplicate (H1299) or triplicate (MCF7) and hits were identified by ranking, based on the relative effect on survival (Suppl. Fig 4 C-F). We identified the top 25 sensitizing siRNAs in each cell line for both control and cisplatin treated conditions (Fig S4C-F; Fig 2I). 16 siRNAs were identified that sensitized both

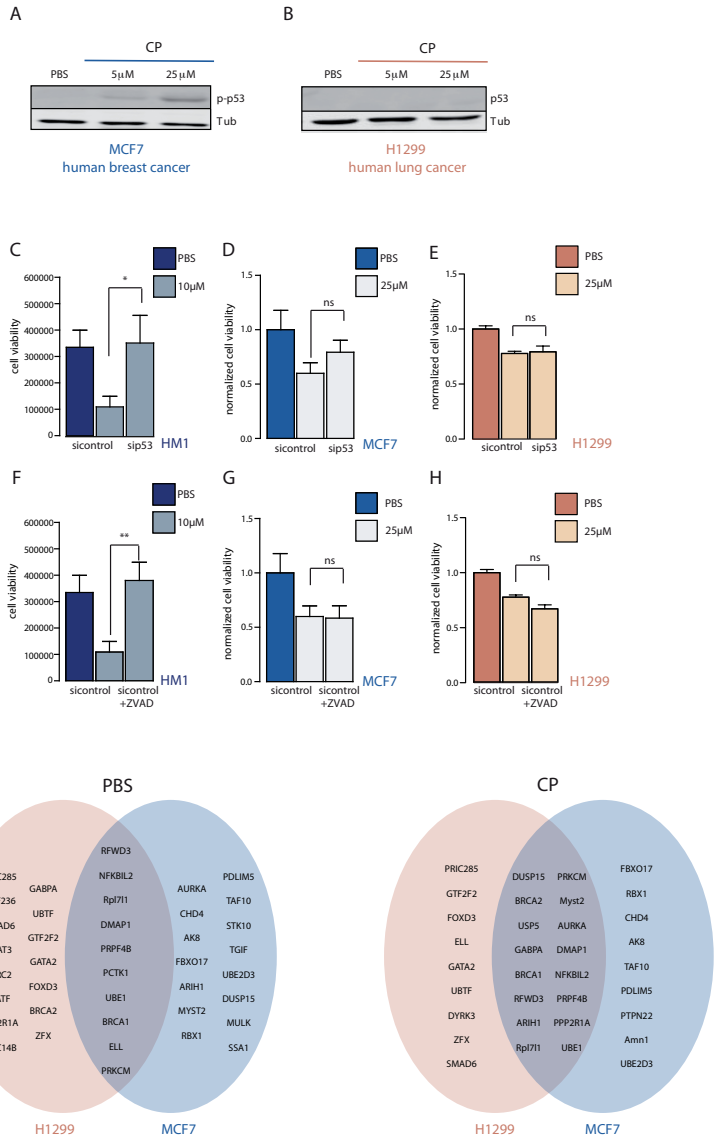

Figure 2. RNAi screens in cisplatin resistant cancer cell lines. (A) WB for p-p53 and Tubulin loading control in MCF7 and (B) for p53 and Tubulin loading in H1299 cells in presence of the indicated cisplatin concentrations. (C-E) Survival of cells treated with indicated concentrations of cisplatin in presence of si-control, sip-53 in (E) HM1 cells (F) MCF7 cells (G) H1299 cells. (F-H) Survival of cells treated with indicated concentrations of cisplatin in presence of si-control or si-control and 100 μM pan-caspase inhibitor ZVAD-fmk in (E) HM1 cells (F) MCF7 cells (G) H1299 cells. (I) Identification of common hits based on loss of survival in PBS and cisplatin conditions in MCF7 and H1299 cells.

MCF7 and H1299 cells to cisplatin (Fig 2I). Of these, 8 siRNAs already reduced survival under control conditions and 8 siRNAs sensitized to cisplatin without significantly affecting basal cell survival in both cell lines simultaneously. DNA repair-related genes, such as the double strand break (DSB) repair factors, BRCA1 and BRCA2; the E1 ubiquitin activating enzyme, Ube1, which has recently been linked to DSB repair; the replication stress- and homologous recombination-related protein NFKBIL2, as well as the single strand break repair-related E3 ligase, Rfdw3; were identified as regulators of survival or (e.g. BRCA2) restricted to regulation of cisplatin-sensitivity [22; 23; 24;25]. General regulators of viability also included DNA methyltransferase 1-associated protein, DMAP1,

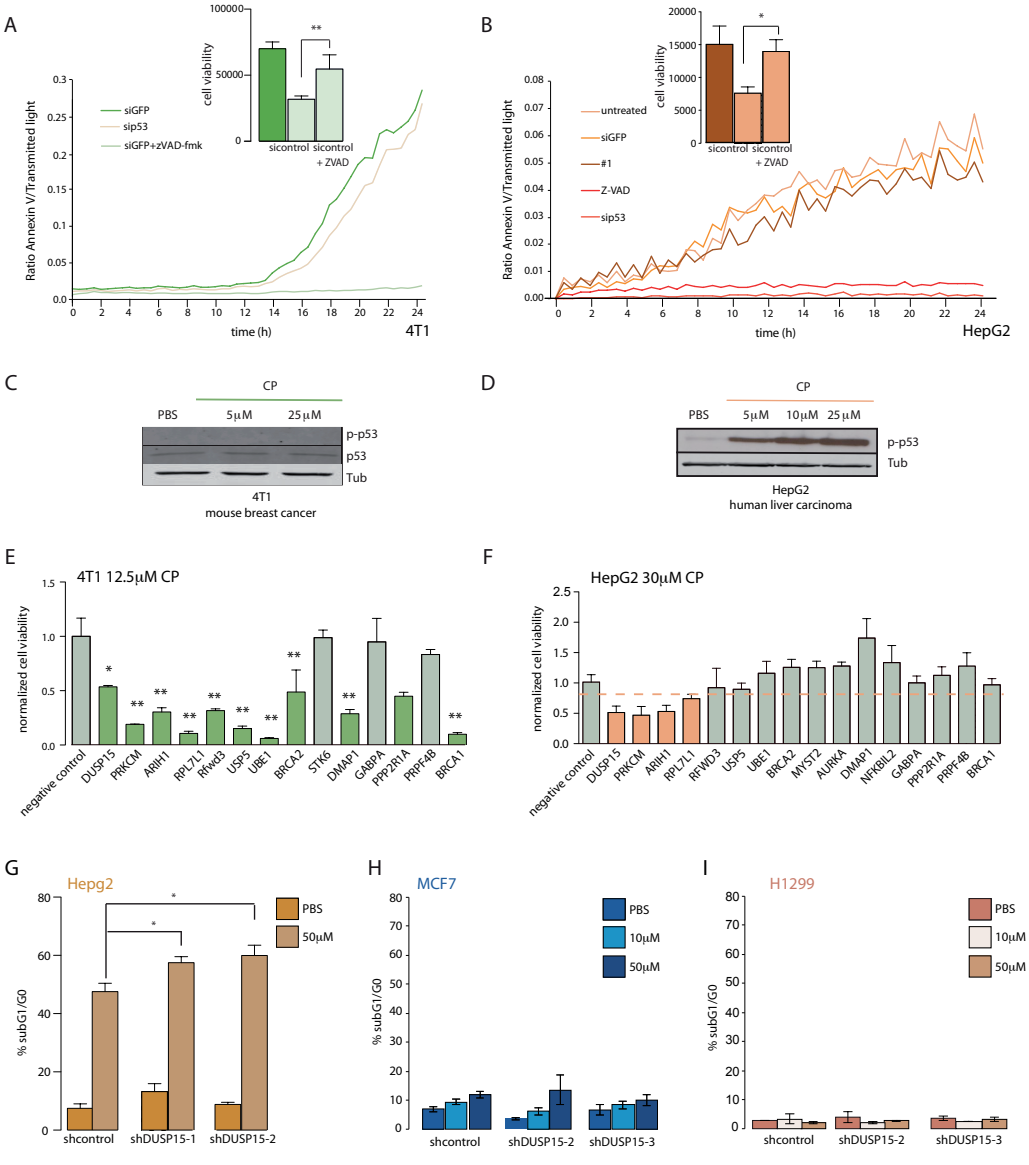

Figure 3. Validation of common sensitizers in intermediately sensitive cancer cell lines. (A, B) Anexin-V labeling of apoptotic cells in presence of si-control or si-control and 100 μM pan-caspase inhibitor ZVAD-fmk or si-p53 in (A) 4T1 cells treated with 12.5 μM cisplatin or (B) HepG2 cells treated with 50 μM cisplatin. Small bar graphs indicate cell survival in 4T1 cells treated for 24 h with 12.5 μM cisplatin (A) or HepG2 cells treated for 24 h with 30 μM cisplatin (B) in the presence of absence of 100 μM ZVAD-fmk. (C) WB for p-p53, p53 and Tubulin loading control in 4T1 cells in the presence of the indicated cisplatin concentrations (D) WB for p-p53 and Tubulin loading control in HepG2 cells in presence of the indicated cisplatin concentrations. (E, F) validation of common cisplatin sensitizers in (E) 4T1 cells treated with 12.5 μM cisplatin or (F) HepG2 cells treated with 30 μM cisplatin for 24 h. (G-I) FACS analysis for subG1/G0 apoptotic fraction of shcontrol and 2 shDUSP15 knockdown cell lines in MCF7 (G), H1299 (H) or HepG2 (I) cells treated for 24 h with the indicated concentrations of cisplatin.

which functions as a transcriptional repressor but also associates with histone acetylase complexes required for recruitment of DNA repair factors [26; 27]; Rpl7l1, a ribosomal protein, whose function has not yet been described; and protein kinase D subunit PRKD1 (PRKCM), which coordinates many cellular functions, such as proliferation or cell motility [28]. Amongst the genes which sensitized both cell lines to cisplatin treatment we found the deubiquitinases USP5, which is involved in recycling of ubiquitin molecules [29; 30] and the E3 ubiquitin ligase ARIH1 [31] as well as the dual specificity phosphatase, DUSP15 [32] and others.

Validation of common hits in two additional cancer cell lines

To further validate siRNAs that sensitized MCF7 and H1299, these were analyzed in 4T1 and HepG2 cell lines (Fig 1A). In contrast to MCF7 and H1299, cisplatin treatment induced apoptosis in 4T1 and HepG2 cells based on i) rescue of cisplatin-induced cell killing by ZVAD-fmk co-treatment (Fig 3A,B), ii) appearance of a subG1/G0 population (Fig S3C,D), and iii) the accumulation of Annexin-V positive cells over time during cisplatin treatment (Fig 3A,B; Fig S5A,B). While induction of apoptosis was caspase-dependent in both HepG2 and 4T1 cells, p53-silencing protected only HepG2 (Fig 3A,B). In agreement, HepG2 cells showed a strong accumulation of active p53 in response to cisplatin treatment while 4T1 cells expressed p53 but showed no response to cisplatin with respect to total or active p53 levels (Fig 3C, D; Fig S5C).

As with MCF7 and H1299 cells, effects of siRNAs were tested using cisplatin concentrations causing ~25% cell death (24 h treatment with 12.5 μM for 4T1; 30 μM for HepG2) (Fig 1B; Fig S2D,E). A large number of hits were confirmed in 4T1 but in HepG2 only four siRNAs appeared to effectively enhance cisplatin-mediated killing (Fig 3E,F). Many of the hits identified in 4T1, similar to their role in H1299 and MCF7 (Fig 2I), already reduced survival under basic conditions (e.g. siRNAs targeting BRCA1, DMAP1, Rfdw3, Ube1, Rpl7l1) (data not shown). Moreover, while siRNA targeting USP5 was a specific cisplatin sensitizer in MCF7 and H1299 (Fig 2I), it already showed significant effects on basal survival of 4T1 cells (data not shown). The four cisplatin-sensitizing siRNAs identified in HepG2 (Fig 3F) did not affect basal HepG2 cell survival (data not shown). Altogether, this pipeline identified siRNAs targeting PRKCM, Rpl7l1, ARIH1, and DUSP15 as cisplatin sensitizers. siRNA targeting ARIH1 or DUSP15 showed only mild reduction of viability in untreated conditions, whereas siRNA targeting Rpl7l1 and PRKCM already killed most cell lines in the absence of cisplatin. As we have recently identified ARIH1 as a mediator of DNA damage-induced translation arrest (von Stechow et al, submitted), here we decided to further study the function of DUSP15 in the cancer cell response to genotoxic stress. We stably expressed lentiviral DUSP15 shRNAs in all three cell lines and in each case selected the independent shRNAs that, after bulk-selection, caused a reduction in DUSP15 mRNA levels of ~70% (Suppl. Fig 6A-C). While the cisplatin-mediated increase in the apoptotic subG1/G0-fraction of the cell cycle was enhanced in HepG2 cells carrying a stable knockdown for DUSP15, no induction of apoptosis was observed in MCF7 or H1299 cells (Fig 3G-I).

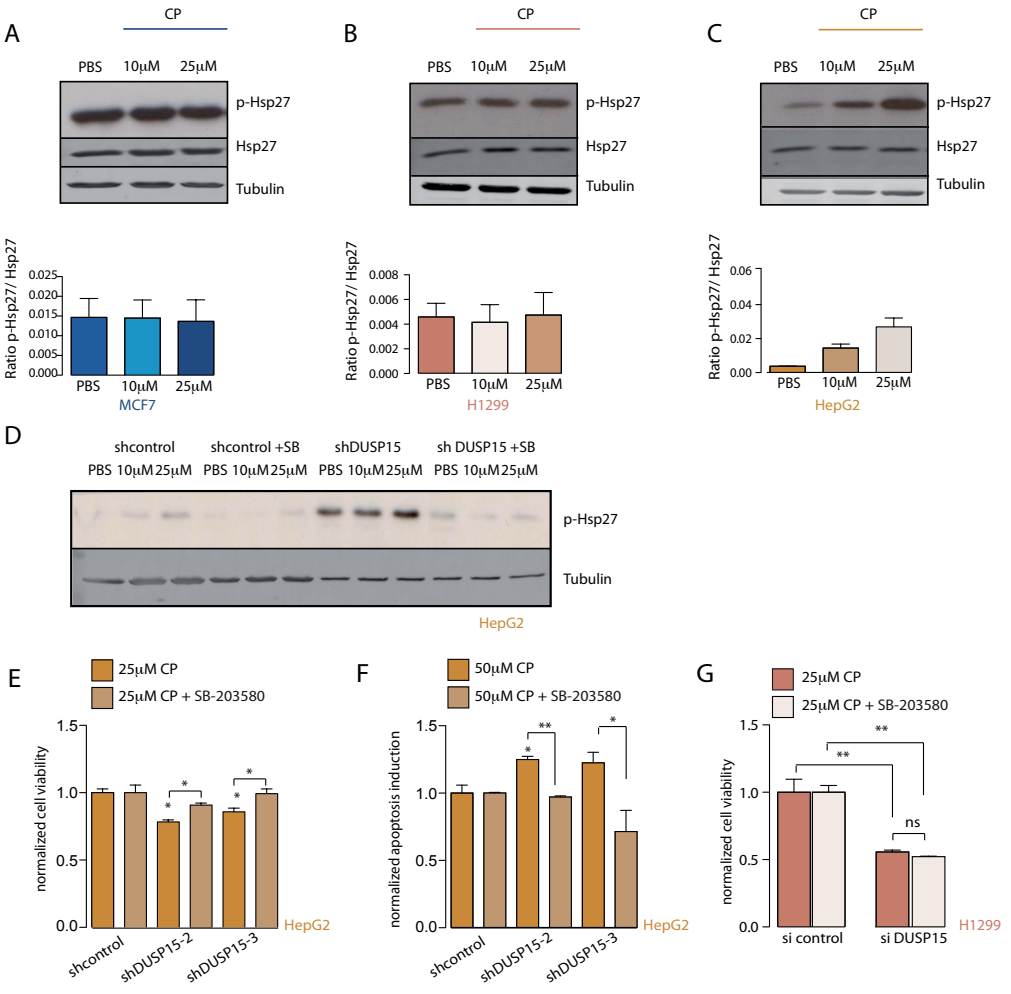

Figure 4. p38 signaling is affected by DUSP15 knockdown in HepG2 but not MCF7 or H1299 cells. (A-C) WB for p-Hsp27, Hsp27 and Tubulin in (A) H1299 (B) MCF7 or (C) HepG2 cells treated with indicated cisplatin concentrations for 4 h. All bands were first corrected for loading using tubulin loading control. Subsequently, ratio of p-HSP27/HSP27 was calculated. (D) WB for p-Hsp27 and Tubulin in shcontrol and 1 shDUSP15 knockdown cell line in HepG2, treated for 4 h with indicated cisplatin conditions, with or without pre-treatment with 10 μM SB203580 p38 inhibitor for 30 min prior to cisplatin exposure (E) cell viability or (F) apoptosis induction in HepG2 und cells, including pre-treatment with 10 μM SB203580 p38 inhibitor for 30 min prior to exposure with indicated cisplatin concentrations (G) cell viability in si-GFP, si-GAPDH or si-DUSP15 treated H1299 cells, after 24 h of treatment with 25 μM cisplatin in presence or absence of 10 μM SB203580 p38 inhibitor (added 30 min prior to cisplatin exposure). All bar graphs represent averages and SEMs of at least three independent experiments.

DUSP15 controls p38MAPK-dependent and independent pathways that regulate the response to cisplatin

DUSP15 belongs to the family of dual-specific phosphatases, which can remove phosphate moieties from Tyrosine as well as Serine/Theronine residues and have been implicated as a negative regulators of MAPK signaling [33]. Recently, p38 MAPKdelta (MK13) was detected as a substrate for DUSP15 in an *in vitro* dephosphorylation assay [34]. To investigate if p38 signaling was affected in response to cisplatin treatment, phosphorylation of Hsp27, a downstream target of the p38-mediated signaling cascade

was analyzed [35]. While levels of p-Hsp27 were high and not responsive to cisplatin in MCF7 and H1299 cells, low basal levels were enhanced upon cisplatin treatment in HepG2 (Fig 4A-C). We wondered if silencing DUSP15 could lead to enhanced p38 signaling in HepG2 cells. Interestingly, basal and cisplatin-induced p-Hsp27 levels were enhanced in HepG2 cells, carrying a stable knockdown for DUSP15 and this effect was blocked by treatment with the p38 inhibitor, SB-203580 [36] (Fig 4D). No effect of DUSP15 shRNAs on p-HSP27 levels was observed in MCF7 or H1299 (data not shown). We next asked if inhibition of p38 signaling could also affect the increased cisplatin sensitivity caused by silencing of DUSP15. Although significant, sensitization to cisplatin in stable DUSP15 shRNA HepG2 cells was weak compared to the effect of transient siRNA-mediated silencing (Fig 3F; Fig 4E). Nevertheless, enhanced cell death and apoptosis observed in cisplatin-treated HepG2 cells in presence of either of the two DUSP15 shRNAs, was reversed by co-treatment with SB-203580 (Fig 4E,F). Lastly, SB-203580 failed to attenuate cisplatin-sensitization in H1299 cells (Fig 4G), where p38 activity appeared not to be modulated by cisplatin (Fig 4B) and where cisplatin-induced reduction of cell numbers was not mediated by apoptosis (Fig 2H; Fig S3A). Taken together, despite the fact that DUSP15 is a potential target for synthetic lethality in the context of cisplatin in a variety of cell types, the underlying mechanisms of action can vary between effects on p38-mediated apoptosis signaling and p38-independent alternative mechanisms of cell death.

DISCUSSION

Different modes of cisplatin-induced cell killing

ES cells, 4T1 mouse breast cancer cells, and HepG2 human liver cancer cells treated with cisplatin display a subG1/G0-fraction in cell cycle profiles and show Annexin V-mediated binding to phsophatidyl serine moved to the outer membrane leaflet [15]. Both events are not necessarily restricted to apoptotic cells, but potentially also to necrotic cells. We verify caspase-mediated apoptosis in both cell lines but the absence of either of these responses in H1299 and MCF7 human cancer cell lines indicates that alternative mechanisms underlie reduced viability after cisplatin treatment. The capability of the caspase-3-deficient breast cancer cell line MCF7 to undergo apoptosis has been debated. Despite reports that MCF7 cells can initiate apoptosis via caspase-9, 7 and 6 dependent routes the actual amount of apoptotic cells observed in these studies remained uncertain [19]. Moreover, other reports argue for a strict dependency of apoptosis on caspase-3 [20] and indicate alternative mechanisms of killing in MCF7 including mitotic catastrophe or autophagic cell death induction [37; 38]. Recent studies showed that apoptosis induction in H1299 cells by γ–irradiation or antineoplastic agents such as roscovitine or resveratrol is dependent on the function of wt p53 [39; 40; 41]. Guanghui et al. showed, that p53 next to inducing apoptosis also counteracts autophagy, which acts as

a pro-survival mechanism in irradiated H1299 cells [39]. Interestingly, despite the fact that in our study 4T1 cells do not accumulate active p53 after cisplatin treatment and are not protected against cisplatin-induced apoptosis by p53 knockdown, the sensitivity of this cell type is much higher than that of H1299 cells, more closely resembling mouse ES cells. This suggests that next to p53 function, other important factors, will determine the cellular response to cisplatin. Our data clearly argue against apoptotic (or necrotic) cell death being induced in H1299 and MCF7 cells and the mechanism of cisplatin-induced cell killing in these cells, may involve senescence, mitotic catastrophe, or autophagy.

Common DNA damage sensitizers

Despite differences in sensitivity and genetic background a few genes sensitize all tested cell lines, either cisplatin specifically, or already under basic condition. The protein kinase D subunit PRKCM is involved in many cancer relevant processes, ranging from proliferation and cell motility to epithelial to mesenchymal transition (EMT), and aberrant expression of this protein has been reported for different types of tumors (e.g. prostate or breast cancer) [28]. Overexpression of PRKCM was shown to increase the proliferation of MCF7 cells in a manner that depends on MEK/ ERK signaling cascades [42]. While knockdown of PRKCM already affects basic survival, some of the reported functions might be important for survival in response to DNA damage, including DNA synthesis and chromatin remodeling, but also detoxification of oxidative stress (which frequently arises as a secondary effect of genotoxic perturbation) [28; 43].

Furthermore, knockdown of the ribosomal protein Rpl7l1 consistently kills cells in control or cisplatin treated conditions. While the function of the ribosomal protein Rpl7l1 has not yet been described, in general ribosomal proteins have been linked to induction p53 signaling in different cases [44]. However, the fact, that knockdown of Rpl7l1 can sensitize the p53 mutant breast cancer cell line 4T1 and the p53-deficient cell line H1299 argues against a mechanism of action for Rpl7l1, which directly involves p53. Moreover, knockdown of Rpl7l1 does not affect p53 levels or cisplatin-induced p53 accumulation in ES cells (data not shown). Despite the lack of knowledge about Rpl7l1 functionality, the strong effect on viability after knockdown indicates a vital role in upholding normal cellular processes. This is undermined by a recent study, which identified Rpl7l1 as a factor necessary for mammalian blastocyste formation [45].

Two siRNAs show selectively enhanced killing in the presence of cisplatin, the E3 ubiquitin ligase, ARIH1 and the dual specific phosphatase, DUSP15. ARIH1 has been identified by us in a separate study, where we have shown that ARIH1 silencing sensitizes through interfering with DNA damage-induced translation arrest (von Stechow et al, submitted).

DUSP15 (also known as VHY) is characterized by a DUSP catalytic domain, and is most closely related to DUSP22 (VHX) [33]. Similar to DUSP22, also DUSP15 can be myristilated, which is a signal for relocation from the cytoplasm to the plasmamembrane [32]. Although DUSP15 expression had been originally described to be restricted to testis, it was recently implicated in oligodendrocyte differentiation, a process that has been

linked to multiple sclerosis [32; 34]. Next to MAPK signaling-related factors, including p38 MAPKdelta (MK13) and the transcription factor ATF2, also PDGFR and SNX6 were identified as potential DUSP15 substrates, in an in vitro dephosphorylation assay [34].

MAPK signaling has been linked to the response to cisplatin on various levels and outcomes have been shown to depend strongly on the cellular context, including both pro- and antiapoptotic functions for all signaling routes, including p38, JNK and MEK/ ERK [46]. In our study, knockdown of DUSP15 affects p38 signaling in HepG2 cells, which initiate p53- and caspase-dependent apoptosis after cisplatin treatment, but not in the "non-apoptotic" cell lines H1299 and MCF7. Whether this is a common theme will require testing a broader panel of cancer cell lines in which this mechanism of cisplatin-induced cell death is intact. Since p53 has been described as a downstream target of p38 signaling [47], the effect of DUSP15 knockdown on apoptosis induction in HepG2 cells may be p53-dependent.

However, an alternative mechanism of DUSP15 knockdown-mediated sensitization seems to be involved for H1299 and MCF7 cells, it remains to be studied how DUSP15 silencing sensitizes other cancer cell types that have corrupted p53 and/ or caspase pathways. The identification of other DUSP15 substrates described above, point to possible modes of action in this respect.

CONCLUSION

Using siRNA screening we were able to identify genes, whose knockdown sensitizes pluripotent stem cells as well as a number of cancer cell lines with varying genetic backgrounds, induced modes of cell killing and cisplatin sensitivities. This panel of genes included a dual specific phosphatase DUSP15, which was shown to act via p38 signaling in p53 and caspase-3 wt cancer cells, but not other cancer cell lines in which cisplatin-induced killing was independent of p53-mediated apoptosis.

MATERIALS & METHODS

Cell culture and materials

HM1 mouse ES cells derived from OLA/129 genetic background (provided by Dr. Klaus Willecke, University of Bonn GE) were maintained under feeder free conditions in GMEM medium containing $5x10^5$ U mouse recombinant leukemia inhibitory factor (LIF; PAA). All other cell lines were purchased from ATCC. MCF7 human breast cancer cells, 4T1 mouse breast cancer cells and H1299 human non-small-cell lung cancer cells were maintained in RPMI medium. HepG2 human liver cancer cells were kept in DMEM. All media contained 10% FBS and 25 U/ml penicillin, and 25 µg/ml streptomycin.

All cell lines, including stable shRNA expressing derivatives, were confirmed to be mycoplasma-free using the Mycosensor kit from Stratagene. For stable gene silencing, cells were transduced using lentiviral TRC shRNA vectors at MOI 1 (LentiExpressTM; Sigma-Aldrich; Dr. Rob Hoeben and Mr Martijn Rabelink, University Hospital, Leiden NL) according to the manufacturers' procedures and bulk selected in medium containing 2.5 µg/ml puromycin. Control vector expressed shRNA targeting TurboGFP.

The DNA cross-linker cisplatin (Cis-PtCl2(NH3)2) (provided by the Pharmacy unit of University Hospital, Leiden NL). The pan-caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk) was purchased from Bachem. The p38 inhibitor SB203580 was from Cell signaling. Antibodies against p53 and phospho-p53 were purchased from Novacostra and Cell signaling, respectively. Antibody against tubulin was obtained from Sigma. Antibodies against p-Hsp27 and Hsp27 were from Cell signaling.

RNAi experiments

siRNAs were purchased from ThermoFisher Scientific. For cancer cell line siRNA screens, customized libraries containing 62 siRNA smartpools cisplatin response modulators from mouse ES cells were used. At least two negative controls were used in RNAi screens. Kif11 siRNA was used as transfection efficiency control. The siRNA screens were performed on a Biomek FX (Beckman Coulter) liquid handling system. 50nM siRNA was transfected in 96 well plates using Dharmafect1 transfection reagent (ThermoFisher Scientific). The medium was refreshed after 24 h and cells were exposed to indicated compounds or vehicle controls 64 h post-transfection for 24 h for 4T1, HepG2 and H1299 cells and for 48 h in MCF7 cells. As readout, a cell viability assay using ATPlite 1Step kit (Perkin Elmer) was performed according to the manufacturer's instructions followed by luminescence measurement using a plate reader for 4T1 cells and H1299 cells. Hoechst staining and cell count was performed for HepG2 cells and MCF7 cells.

RNAi screen data analysis

To rank the results, relative amount of killing were determined and hits were ranked by effect on survival and significance. The top 25 hits in each cell line were overlaid in Venn-Diagrams and common hits were identified.

Apoptosis analysis

Cells were exposed for indicated times and cisplatin concentrations for apoptosis analysis. Floating and attached cells were pooled and fixed in 80% ethanol overnight. Cells were stained using PBS EDTA containing 7.5 mM propidium iodine and 40 mg/ml RNAseA and measured by flow cytometry (FACSCanto II; Becton Dickinson). The amount of cells in the different cell cycle fractions or in sub G0/G1 for apoptotic cells was calculated using BD FACSDiva software. Alternatively, apoptosis was determined using live imaging of Annexin V-labeling, as described previously [48].

Western blot analysis

Extracts were prepared in TSE containing protein inhibitor cocktail and separated by SDS-PAGE on polyacrylamide gels, transferred to PVDF membranes, and membranes were blocked using 5% BSA. Following incubation with primary and secondary antibodies signal was detected using a Typhoon™ 9400 from GE Healthcare.

Immunofluorescence

HM1 mouse ES cells and 4T1 breast cancer cells were seeded in 96 well µclear plates from Greiner. Subsequently, they were treated with cisplatin and fixed using 4% formaldehyde for 10 min. After extensive washing and permeabilization with 0.05% TritonX, cells were stained with p-p53 antibody, followed by counterstaining with DAPI and appropriate secondary fluorescent antibody

qPCR

RNA was extracted using RNeasy Plus Mini Kit from Qiagen. cDNA was made from 50 ng total RNA with RevertAid H minus First strand cDNA synthesis kit (Fermentas) and real-time qPCR was subsequently performed in triplicate using SYBR green PCR (Applied Biosystems) on a 7900HT fast real-time PCR system (Applied Biosystems). The following qPCR primer sets were used: GAPDH, forward (fw) AGCCACATCGCTCAGACACC reverse (rev) ACCCGTTGACTCCGACCTT; DUSP15 (fw). CACTGCTTTGCAGGCATCTC (rev) GCCCCGTCACAGTCATCAC. Data were collected and analyzed using SDS2.3 software (Applied Biosystems). Relative mRNA levels after correction for GAPDH control mRNA were expressed using 2^(-ΔΔCt) method.

REFERENCES

1. Helleday, T., Petermann, E., Lundin, C., Hodgson, B. & Sharma, R. A. (2008). DNA repair pathways as targets for cancer therapy. Nat Rev Cancer 8, 193-204.

2. Lord, C. J. & Ashworth, A. (2012). The DNA damage response and cancer therapy. Nature 481, 287-94.

3. Bouwman, P. & Jonkers, J. (2012). The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. Nat Rev Cancer 12, 587-98.

4. Jackson, S. P. & Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071-8.

5. d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer 8, 512-22.

6. Soussi, T. & Lozano, G. (2005). p53 mutation heterogeneity in cancer. Biochem Biophys Res Commun 331, 834-42.

7. Vousden, K. H. & Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell 137, 413-31.

8. Muller, P. A. & Vousden, K. H. (2013). p53 mutations in cancer. Nat Cell Biol 15, 2-8.

9. Vermeulen, K., Van Bockstaele, D. R. & Berneman, Z. N. (2005). Apoptosis: mechanisms and relevance in cancer. Ann Hematol 84, 627-39.

10. Cotter, T. G. (2009). Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 9, 501-7.

11. Singh, R., George, J. & Shukla, Y. (2010). Role of senescence and mitotic catastrophe in cancer therapy. Cell Div 5, 4.

12. Jackson, J. G., Pant, V., Li, Q., Chang, L. L., Quintas-Cardama, A., Garza, D., Tavana, O., Yang, P., Manshouri, T., Li, Y., El-Naggar, A. K. & Lozano, G. (2012). p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. Cancer Cell 21, 793-806.

13. Reinhardt, H. C., Aslanian, A. S., Lees, J. A. & Yaffe, M. B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell 11, 175-89.

14. Jiang, H., Reinhardt, H. C., Bartkova, J., Tommiska, J., Blomqvist, C., Nevanlinna, H., Bartek, J., Yaffe, M. B. & Hemann, M. T. (2009). The combined status of ATM and p53 link tumor development with therapeutic response. Genes Dev 23, 1895-909.

15. Carreras Puigvert, J., von Stechow, L., Siddappa, R., Pines, A., Bahjat, M., Haazen, L. C., Olsen, J. V., Vrieling, H., Meerman, J. H., Mullenders, L. H., van de Water, B. & Danen, E. H. (2013). Systems biology approach identifies the kinase csnk1a1 as a regulator of the DNA damage response in embryonic stem cells

Sci. Signal 6, ra5.

16. Pines, A., Kelstrup, C. D., Vrouwe, M. G., Puigvert, J. C., Typas, D., Misovic, B., de Groot, A., von Stechow, L., van de Water, B., Danen, E. H., Vrieling, H., Mullenders, L. H. & Olsen, J. V. (2011). Global phosphoproteome profiling reveals unanticipated networks responsive to cisplatin treatment of embryonic stem cells. Mol Cell Biol 31, 4964-77.

17. Ben-David, U. & Benvenisty, N. (2011). The tumorigenicity of human embryonic and induced pluripotent stem cells. Nat Rev Cancer 11, 268-77.

18. Rothenberg, M. E., Clarke, M. F. & Diehn, M. (2010). The Myc connection: ES cells and cancer. Cell 143, 184-6.

19. Liang, Y., Yan, C. & Schor, N. F. (2001). Apoptosis in the absence of caspase 3. Oncogene 20, 6570-8.

20. Blanc, C., Deveraux, Q. L., Krajewski, S., Janicke, R. U., Porter, A. G., Reed, J. C., Jaggi, R. & Marti, A. (2000). Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 60, 4386-90.

21. Li, Z., Musich, P. R. & Zou, Y. (2011). Differential DNA damage responses in p53 proficient and deficient cells: cisplatin-induced nuclear import of XPA is independent of ATR checkpoint in p53-deficient lung cancer cells. Int J Biochem Mol Biol 2, 138-145.

22. O'Connell, B. C., Adamson, B., Lydeard, J. R., Sowa, M. E., Ciccia, A., Bredemeyer, A. L., Schlabach, M., Gygi, S. P., Elledge, S. J. & Harper, J. W. (2010). A genome-wide camptothecin sensitivity screen identifies a mammalian MMS22L-NFKBIL2 complex required for genomic stability. Mol Cell 40, 645-57.

23. Roy, R., Chun, J. & Powell, S. N. (2012). BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 12, 68-78.

24. Liu, S., Chu, J., Yucer, N., Leng, M., Wang, S. Y., Chen, B. P., Hittelman, W. N. & Wang, Y. (2011). RING finger and WD repeat domain 3 (RFWD3) associates with replication protein A (RPA) and facilitates RPA-mediated DNA damage response. J Biol Chem 286, 22314-22.

25. Moudry, P., Lukas, C., Macurek, L., Hanzlikova, H., Hodny, Z., Lukas, J. & Bartek, J. (2012). Ubiquitin-activating enzyme UBA1 is required for cellular response to DNA damage. Cell Cycle 11, 1573-82.

26. Penicud, K. & Behrens, A. (2013). DMAP1 is an essential regulator of ATM activity and function. Oncogene.

27. Lee, G. E., Kim, J. H., Taylor, M. & Muller, M. T. (2010). DNA methyltransferase 1-associated protein (DMAP1) is a co-repressor that stimulates DNA methylation globally and locally at sites of double strand break repair. J Biol Chem 285, 37630-40.

28. Sundram, V., Chauhan, S. C. & Jaggi, M. (2011). Emerging roles of protein kinase D1 in cancer. Mol Cancer Res 9, 985-96.

29. Avvakumov, G. V., Walker, J. R., Xue, S., Allali-Hassani, A., Asinas, A., Nair, U. B., Fang, X., Zuo, X., Wang, Y. X., Wilkinson, K. D. & Dhe-Paganon, S. (2012). Two ZnF-UBP domains in isopeptidase T (USP5). Biochemistry 51, 1188-98.

30. Dayal, S., Sparks, A., Jacob, J., Allende-Vega, N., Lane, D. P. & Saville, M. K. (2009). Suppression of the deubiquitinating enzyme USP5 causes the accumulation of unanchored polyubiquitin and the activation of p53. J Biol Chem 284, 5030-41.

31. Tan, N. G., Ardley, H. C., Rose, S. A., Leek, J. P., Markham, A. F. & Robinson, P. A. (2000). Characterisation of the human and mouse orthologues of the Drosophila ariadne gene. Cytogenet Cell Genet 90, 242-5.

32. Alonso, A., Narisawa, S., Bogetz, J., Tautz, L., Hadzic, R., Huynh, H., Williams, S., Gjorloff-Wingren, A., Bremer, M. C., Holsinger, L. J., Millan, J. L. & Mustelin, T. (2004). VHY, a novel myristoylated testis-restricted dual specificity protein phosphatase related to VHX. J Biol Chem 279, 32586-91.

33. Patterson, K. I., Brummer, T., O'Brien, P. M. & Daly, R. J. (2009). Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J 418, 475-89.

34. Schmidt, F., van den Eijnden, M., Pescini Gobert, R., Saborio, G. P., Carboni, S., Alliod, C., Pouly, S., Staugaitis, S. M., Dutta, R., Trapp, B. & Hooft van Huijsduijnen, R. (2012). Identification of VHY/Dusp15 as a regulator of oligodendrocyte differentiation through a systematic genomics approach. PLoS One 7, e40457.

35. Sarkar, D., Su, Z. Z., Lebedeva, I. V., Sauane, M., Gopalkrishnan, R. V., Valerie, K., Dent, P. & Fisher, P. B. (2002). mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by

inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. Proc Natl Acad Sci U S A 99, 10054-9.

36. Zer, C., Sachs, G. & Shin, J. M. (2007). Identification of genomic targets downstream of p38 mitogen-activated protein kinase pathway mediating tumor necrosis factor-alpha signaling. Physiol Genomics 31, 343-51.

37. Morse, D. L., Gray, H., Payne, C. M. & Gillies, R. J. (2005). Docetaxel induces cell death through mitotic catastrophe in human breast cancer cells. Mol Cancer Ther 4, 1495-504.

38. Bursch, W., Karwan, A., Mayer, M., Dornetshuber, J., Frohwein, U., Schulte-Hermann, R., Fazi, B., Di Sano, F., Piredda, L., Piacentini, M., Petrovski, G., Fesus, L. & Gerner, C. (2008). Cell death and autophagy: cytokines, drugs, and nutritional factors. Toxicology 254, 147-57.

39. Guanghui, C., Dejuan, K., Xue, H., Bing, L., Mengzi, H., Nan, L., Shumei, M. & Xiaodong, L. (2012). The Tumor Suppressor, p53, Contributes to Radiosensitivity of Lung Cancer Cells by Regulating Autophagy and Apoptosis. Cancer Biother Radiopharm.

40. Slovackova, J., Smarda, J. & Smardova, J. (2012). Roscovitine-induced apoptosis of H1299 cells depends on functional status of p53. Neoplasma 59, 606-12.

41. Ferraz da Costa, D. C., Casanova, F. A., Quarti, J., Malheiros, M. S., Sanches, D., Dos Santos, P. S., Fialho, E. & Silva, J. L. (2012). Transient transfection of a wild-type p53 gene triggers resveratrol-induced apoptosis in cancer cells. PLoS One 7, e48746.

42. Karam, M., Legay, C., Auclair, C. & Ricort, J. M. (2012). Protein kinase D1 stimulates proliferation and enhances tumorigenesis of MCF-7 human breast cancer cells through a MEK/ERK-dependent signaling pathway. Exp Cell Res 318, 558-69.

43. Galluzzi, L., Senovilla, L., Vitale, I., Michels, J., Martins, I., Kepp, O., Castedo, M. & Kroemer, G. (2012). Molecular mechanisms of cisplatin resistance. Oncogene 31, 1869-83.

44. Macias, E., Jin, A., Deisenroth, C., Bhat, K., Mao, H., Lindstrom, M. S. & Zhang, Y. (2010). An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. Cancer Cell 18, 231-43.

45. Maserati, M., Dai, X., Walentuk, M. & Mager, J. (2012). Identification of four genes required for mammalian blastocyst formation. Zygote, 1-9.

46. Brozovic, A. & Osmak, M. (2007). Activation of mitogen-activated protein kinases by cisplatin and their role in cisplatin-resistance. Cancer Lett 251, 1-16.

47. Wu, G. S. (2004). The functional interactions between the p53 and MAPK signaling pathways. Cancer Biol Ther 3, 156-61.

48. Puigvert, J. C., de Bont, H., van de Water, B. & Danen, E. H. (2010). High-throughput live cell imaging of apoptosis. Curr Protoc Cell Biol Chapter 18, Unit 18 10 1-13.

SUPPLEMENTARY MATERIALS

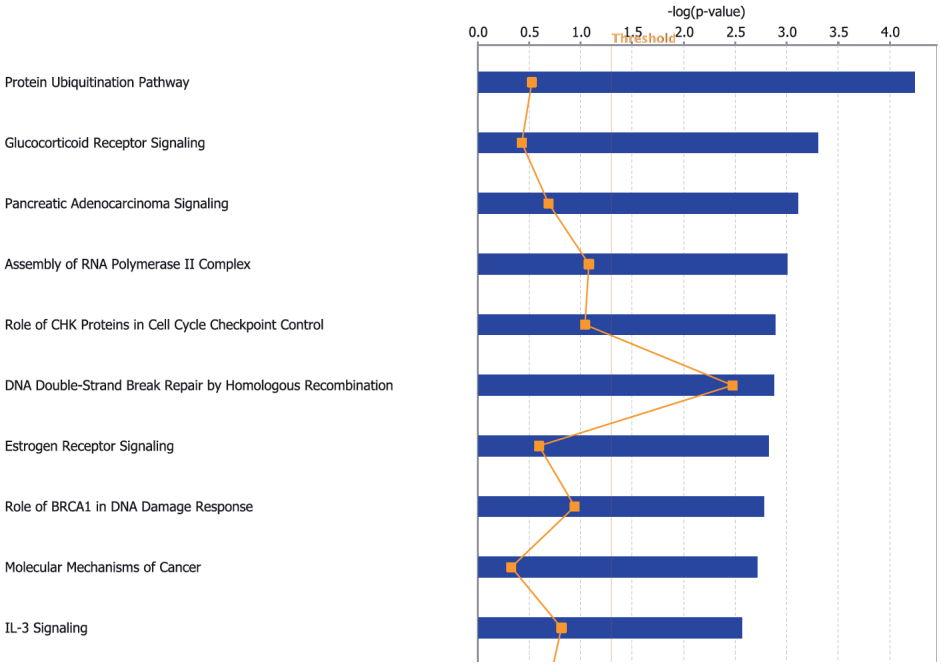

Figure S1. IPA pathway analysis on 62 ES cell sensitizers.

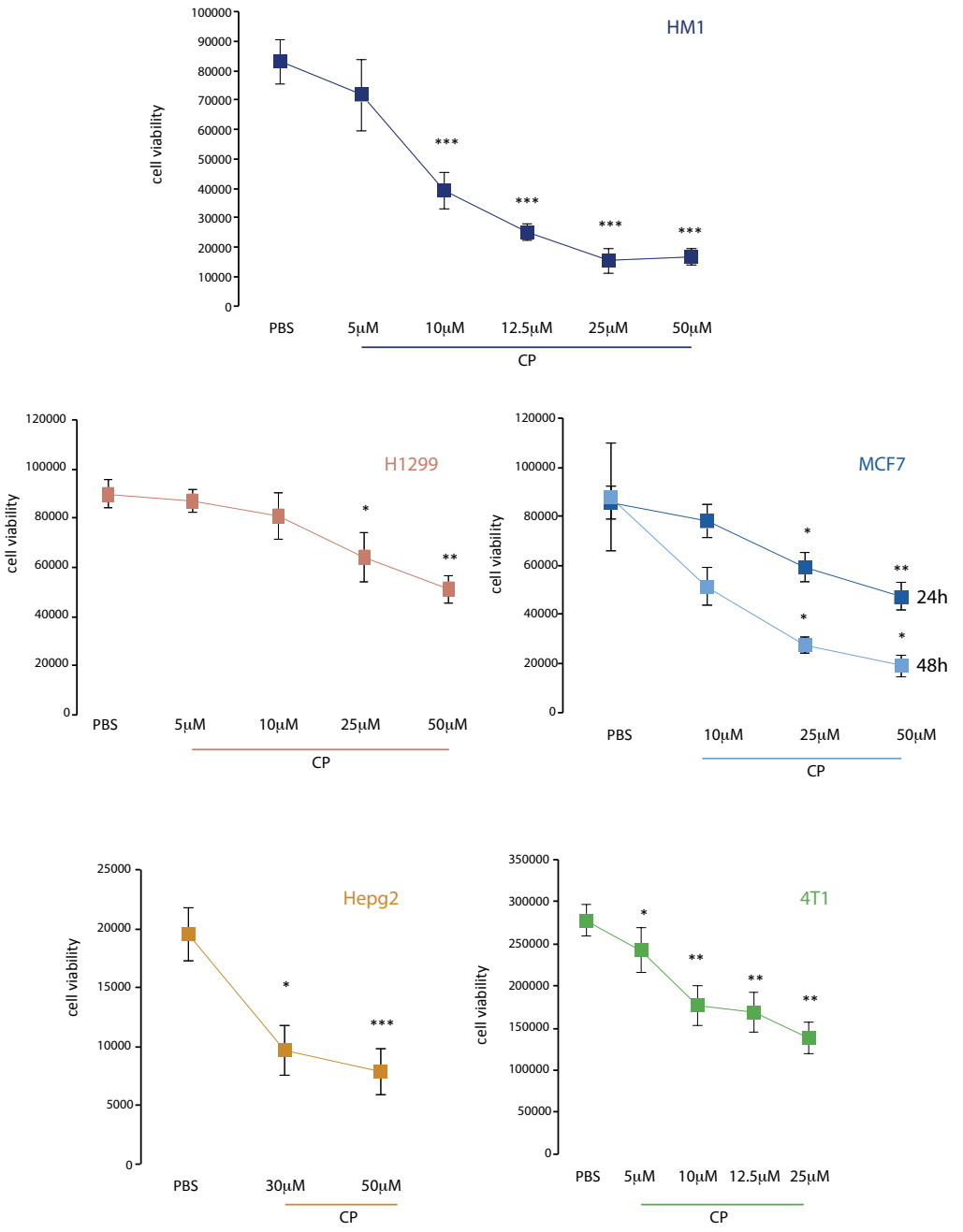

Figure S2. Cell lines vary in cisplatin sensitivity. Cell survival in HM1, H1299, MCF7, HepG2 and 4T1 cells treated with indicated cisplatin concentrations for 24 h or 48 h. Line graphs indicate means and SEMs of at least three independent experiments.

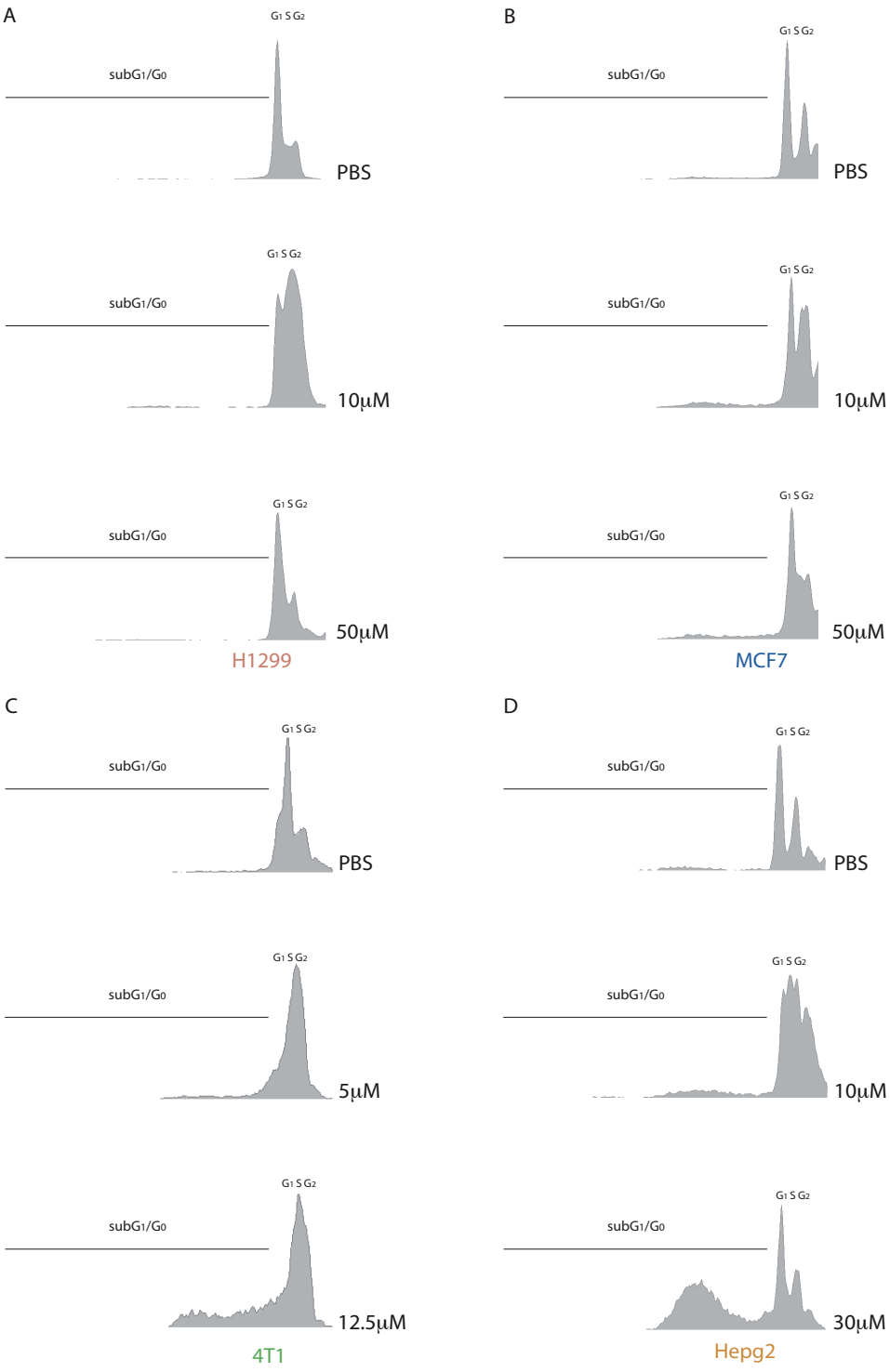

Figure S3. Apoptotic cell fractions after cisplatin treatment. Profiles of cell cycle distribution and subG1/G0 apoptotic fraction of the cell cycle in (A) H1299 cells (treated for 48 h) (B) MCF7 cells (treated for 48 h) (C) 4T1 cells (treated for 24 h) (D) HepG2 (treated for 24 h) cells in the presence of indicated cisplatin concentrations.

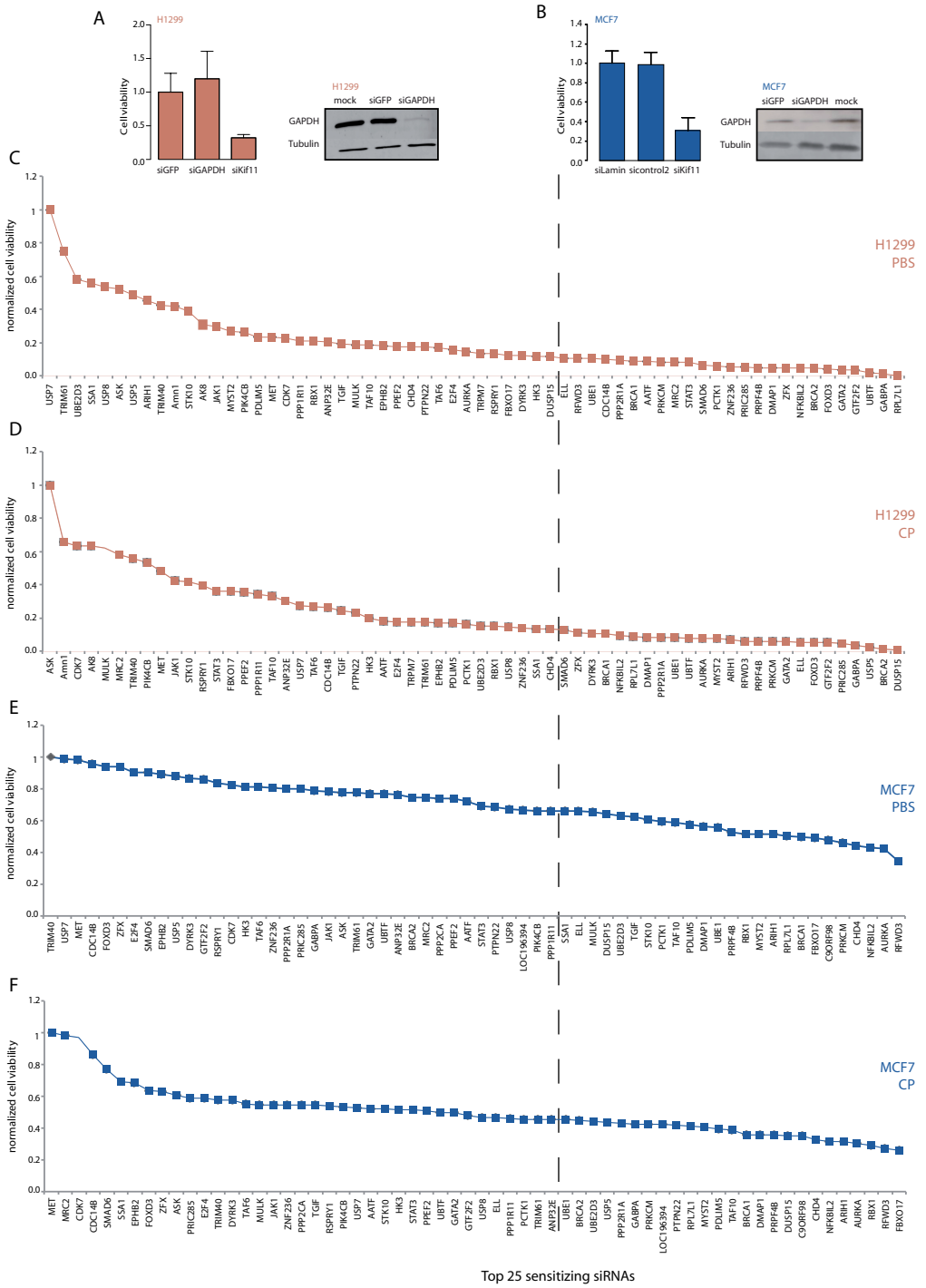

Figure S4. RNAi screens in H1299 and MCF7 cells. (A, B) Normalized cell viability in MCF7 (A) and H1299 (B) cells treated with two different control siRNAs and siRNA targeting Kif11, indicating transfection efficiency; WB for GAPDH and tubulin loading control in MCF7 and H1299 cells treated with only transfection reagent (mock) or siRNA targeting GFP or GAPDH indicating knockdown efficiency (C-F) Cell viability in H1299 (C, D) and MCF7 (normalized to negative controls, siRNA with the highest relative survival is set to 1) (E, F) cells under PBS and 25 μM cisplatin treated conditions, in presence of indicated siRNAs, ranked for their effect on cell survival. Dotted line indicates top 25 sensitizing siRNAs.

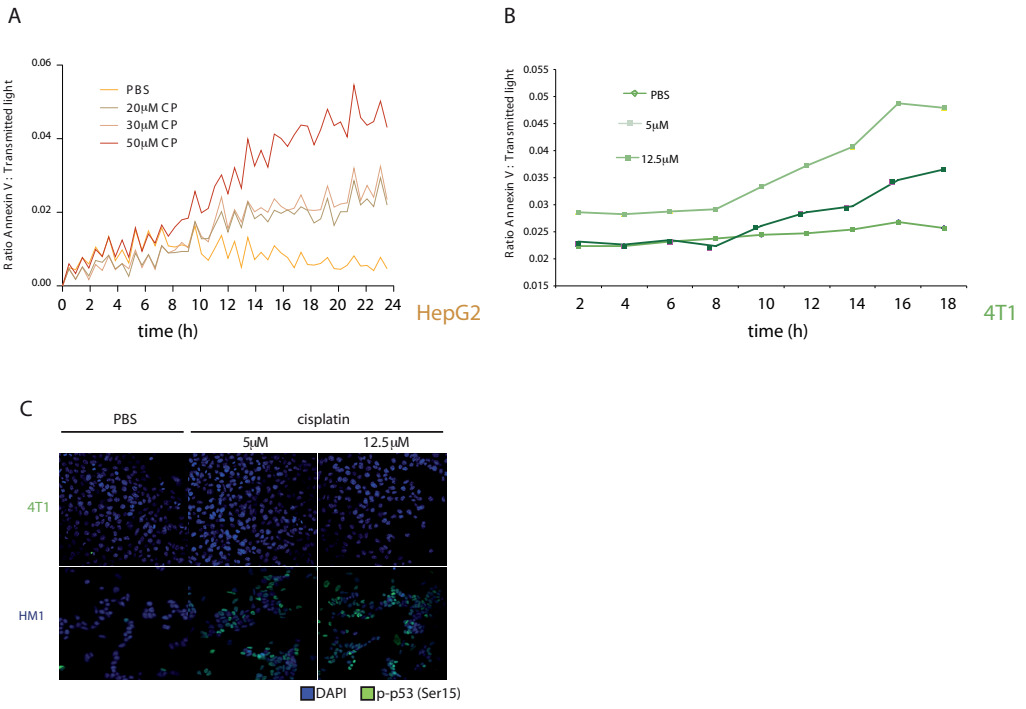

Figure S5. Mode of killing in HepG2 and 4T1 cells. (A, B) Anexin-labeling of apoptotic cells after treatment with indicated cisplatin concentrations in (A) HepG2 and (B) 4T1 cells. (C) Immunostaining for p-p53 in HM1 and 4T1 cells after treatment with the indicated concentrations of cisplatin

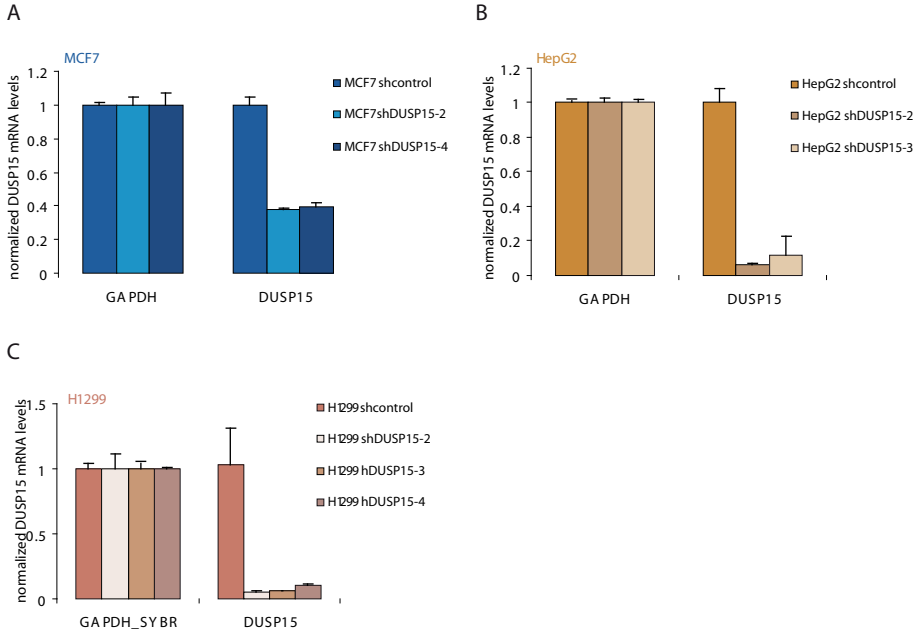

Figure S6. DUSP15 knockdown in different cancer cell lines. (A-C) mRNA expression of DUSP15 normalized to GAPDH after stable expression of shRNA of shcontrol and at least 2 shDUSP15 knockdown cell lines in MCF7 (A) Hepg2 (B) and H1299 (C) cells.