Oncogene (2005) 24, 4572–4579

www.nature.com/onc

# Stress-specific signatures: expression profiling of p53 wild-type and -null human cells

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**Gene expression responses of human cell lines exposed to a diverse set of stress agents were compared by cDNA microarray hybridization. The B-lymphoblastoid cell line TK6 (p53 wild-type) and its p53-null derivative, NH32, were treated in parallel to facilitate investigation of p53-dependent responses. RNA was extracted 4 h after the beginning of treatment when no notable decrease in cell viability was evident in the cultures. Gene expression signatures were defined that discriminated between four broad general mechanisms of stress agents: Non-DNA-damaging stresses (heat shock, osmotic shock, and 12-*O*-tetradecanoylphorbol 13-acetate), agents causing mainly oxidative stress (arsenite and hydrogen peroxide), ionizing radiations (neutron and γ-ray exposures), and other DNA-damaging agents (ultraviolet radiation, methyl methanesulfonate, adriamycin, camptothecin, and *cis*-Platinum(II)diammine dichloride (cisplatin)). Within this data set, non-DNA-damaging stresses could be discriminated from all DNA-damaging stresses, and profiles for individual agents were also defined. While DNA-damaging stresses showed a strong p53-dependent element in their responses, no discernible p53-dependent responses were triggered by the non-DNA-damaging stresses. A set of 16 genes did exhibit a robust p53-dependent pattern of induction in response to all nine DNA-damaging agents, however.**

*Oncogene* (2005) **24,** 4572–4579. doi:10.1038/sj.onc.1208653
Published online 11 April 2005

**Keywords:** cDNA microarray; DNA damage; heavy metals; p53

## Introduction

It is now generally accepted that the eucaryotic cellular response to stress is complex and can involve altered regulation of a considerable fraction of the transcribed genome. For instance, up to 20% of the yeast genome may be stress responsive (Jelinsky *et al*., 2000). There is great interest in exploiting gene expression responses to streamline toxicological testing and to predict mechanisms of action of new agents. Initial steps in this direction include defining gene expression signatures for different classes of toxic agents. For example, a gene expression signature was identified discriminating between ionizing radiation and base-damaging agents, such as ultraviolet (UV) radiation and cisplatin (Park *et al*., 2002). Another profile identified cisplatin as distinct from nongenotoxic agents (Burczynski *et al*., 2000), while a study using rats treated *in vivo* identified a gene expression profile distinguishing between pheonbarbital and peroxisome proliferators (Hamadeh *et al*., 2002a, b).

The present study compares agents representing several diverse mechanisms of initial action. Nongenotoxic stresses that do not cause initial damage to DNA include osmotic shock, heat shock, and 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Hyperosmolality has been shown to induce genes such as *GADD45A* and *DDIT3* through activation of protein kinase cascades (Kultz *et al*., 1998) and proteins including molecular chaperones and transmembrane transporters (Burg *et al*., 1996). Heat shock also places stress on the protein folding apparatus of the cell, and results in the induction of molecular chaperones, proteases and transcription factors (Jolly and Morimoto, 2000). TPA is a tumor promoter that activates protein kinase C and mitogen-activated protein (MAP) kinases (McClellan *et al*., 1999) and can induce apoptosis or differentiation in some leukemia cell lines (Shimizu *et al*., 1991).

As a model genotoxic agent, ionizing radiation is a useful probe of cellular stress responses, in part because its physical properties allow precise dosimetry free from issues of chemical uptake and metabolism. We have previously characterized many aspects of the gene expression profiles produced by ionizing radiation (Amundson *et al*., 1999, 2000, 2003). While our studies to date have focused on low linear energy transfer (LET)

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Received 21 December 2004; revised 29 January 2005; accepted 4 February 2005; published online 11 April 2005

$\gamma$-ray exposures, this study also employs high LET neutron exposures. High LET ionizing radiation produces unique types of complex clustered DNA damage, which is relatively resistant to repair processes (Goodhead, 1989). Ionizing radiation in general produces DNA damage including double-strand breaks, and a variety of oxidative lesions. Two other agents in this study, hydrogen peroxide ($H_2O_2$) and sodium arsenite, act primarily through generation of oxidative stress, but without causing direct double-strand breaks. Arsenite affects the expression of many genes, and has been reported to inhibit NF-kB and enhance AP-1 and p53 activity (Beyersmann, 2002).

The remaining DNA-damaging agents cause mainly base damage and other lesions toxic in S phase. They include UV radiation (UVB), Methyl methanesulfonate (MMS), and the chemotherapy agents *cis*-Platinum(II)-diammine dichloride (cisplatin), adriamycin, and camptothecin (CPT). UVB, comprising the 280–320 nm part of the UV spectrum, generates reactive oxygen species and well-defined DNA lesions, such as pyrimidine dimers and pyrimidine–pyrimidone (6-4) photoproducts. UVB irradiation activates stress signaling pathways, including p53 and MAP kinase, resulting in regulation of many genes (Koch-Paiz *et al*., 2004). Gene induction patterns in response to MMS have some similarities to those induced by UV (Zhan *et al*., 1996b), although MMS induces alkyl lesions in DNA, proteins, and other cellular macromolecules. Cisplatin is another alkylating agent, which induces interstrand and intrastrand crosslinks in DNA and disrupts cytoskeletal proteins (Kopf-Maier and Muhlhausen, 1992). Adriamycin (or doxorubicin) is an antibiotic that damages DNA as an intercalating agent. Finally, CPT is a topoisomerase I inhibitor, which produces single-strand DNA breaks by stabilizing the topoisomerase I interaction with single-strand cleavable complexes and halting the normal reaction. The resulting complexes also interfere with replication, causing S-phase-specific cell cycle arrest and toxicity.

The tumor suppressor gene p53 plays a major role in modulating gene expression responses to many stresses, and its downstream effector genes play roles in diverse cellular processes including apoptosis, DNA repair, cell cycle arrest, and senescence. The role of p53 in the cellular response to ionizing radiation has been well characterized, and varies among different cell types. For instance, early radiation-induced apoptosis occurs mainly in cells of myeloid and lymphoid lineages, frequently correlated with specific p53-dependent gene induction (Zhan *et al*., 1996a). Many of these apoptosis-associated genes are also regulated by MAPK pathways. Since the MAPKs p38 and JNK phosphorylate p53 at regulatory sites (Bulavin *et al*., 1999; Hildesheim *et al*., 2002), this illustrates an opportunity for interpathway crosstalk. Hence, p53 signaling can be shaped and regulated differently depending on both cellular context, and the activation of other signaling pathways by a specific stress agent. Such interpathway crosstalk may contribute to the regulation of genes such as *GADD45A*, which is strictly dependent on wild-type p53 for induction by ionizing radiation, but which responds to UV radiation or MMS exposure through both p53-dependent and -independent pathways (Zhan *et al*., 1996b). Thus, interactions between signaling pathways will shape the gene expression responses and ultimate outcome of exposure to different stress agents.

The p53 wild-type TK6 cell line has been extensively characterized and employed in numerous toxicology studies with cytotoxic and mutational end points (Kelsey *et al*., 1994; Steen *et al*., 1997; Zhan *et al*., 2004). We have also found it to be among the most robust responders to ionizing radiation in terms of gene regulation. The availability of NH32, a targeted p53 knockout cell line derived from TK6 (Chuang *et al*., 1999), enables studies targeted at this key signaling pathway. The investigation of the role of p53 in shaping responses to diverse stress agents represents a novel dimension of the present study, which for the first time compares the genomic responses of two closely related human cell lines differing only in p53 status after exposure to 12 diverse stress agents. The expression of many genes was either up- or downregulated in response to one or more of the treatments, and multiple gene expression signatures were discernable. Different sets of genes could discriminate between genotoxic and non-genotoxic stresses, or define subcategories of mechanisms of action within each class. Signatures for TPA, ionizing radiation, and heavy metal exposures were explored. Surprisingly, these signatures were independent of cellular p53 status, although a separate signature discriminating p53 status was also contained within the gene expression responses to the DNA-damaging agents. In contrast, there was no evidence for participation of p53 in the global gene expression response to nonDNA-damaging stresses.

## Results

### *Initial toxicity and gene expression*

The stress treatments used in this study are summarized in Table 1, and gene expression was assayed 4 h after the beginning of treatment. We have previously found this incubation time to allow maximum expression of early response genes following ionizing radiation stress, and robust expression of *CDKN1A* was observed by this time for most of the treatments in this study (Table 2). Additionally, hybridization to *HSPA1L* was used to monitor heat-shock responses, and *EGR1* for TPA. Further experiments of dose and time dependence of gene induction were performed for several of the agents (data not shown) to assist in selecting optimal doses and to ensure that the 4-h time-point was appropriate. None of the treatments had a marked effect on viability of either cell line by the end of the 4-h incubation period as measured by trypan blue exclusion (Figure 1). The 4-h incubation thus avoids possible effects stemming from late toxicity, which could complicate interpretation and comparison of the gene expression profiles. (Analysis of

**Table 1** Stress agents

| *Treatment* | *Dose* | *Agent type* |
|---|---|---|
| $\gamma$-Rays | 2.5 Gy | Ionizing radiation |
| $\gamma$-Rays | 8 Gy | Ionizing radiation |
| Neutrons | 0.5 Gy | Ionizing radiation |
| UVB | 100 J/m$^2$ | Genotoxic |
| Adriamycin | 200 ng/ml | Genotoxic |
| MMS | 50 $\mu$g/ml | Genotoxic |
| Cisplatin | 30 $\mu$M | Genotoxic |
| Camptothecin | 250 nM | Genotoxic |
| TPA | 200 nM | Nongenotoxic |
| NaCl | 500 mOsM | Nongenotoxic |
| Heat–shock | 20 min/47°C | Nongenotoxic |
| $H_2O_2$ | 200 $\mu$M | Oxidative stress |
| Arsenic | 30 $\mu$M | Oxidative/metal |
| $CdCl_2$[a] | 50 $\mu$M | Oxidative/metal |

[a]$CdCl_2$ treatments were performed after the initial analysis of the other treatments

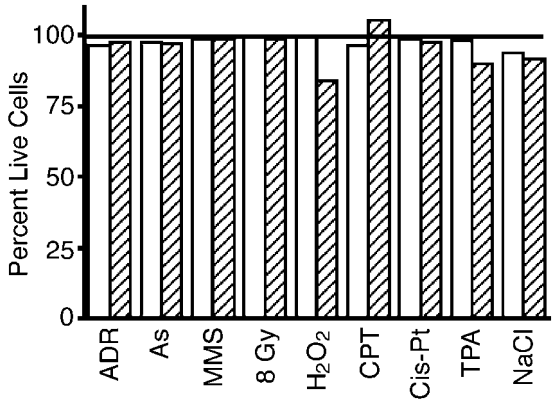


**Figure 1** Surviving fractions in treated cultures determined by trypan blue exclusion normalized to untreated controls. At least 250 cells were scored 4 h after treatment. White bars: TK6, hatched bars: NH32

**Table 2** Gene induction 4 h after treatment

| *Treatment* | *Gene* | *TK6* | | *NH32* | |
|---|---|---|---|---|---|
| | | *Dot blot* | *Microarray* | *Dot blot* | *Microarray* |
| $\gamma$-Ray (8 Gy) | *CDKN1A* | 12.7 | 11.2 | 1.4 | 1.4 |
| UVB | *CDKN1A* | 7.9 | 5.4 | 1.9 | 1.5 |
| Adriamycin | *CDKN1A* | 9.2 | 8.6 | 1.2 | 1.6 |
| MMS | *CDKN1A* | 9.8 | 10.3 | 1.7 | 2.6 |
| Cisplatin | *CDKN1A* | 2.7 | 3.8 | 1.6 | 1.5 |
| Camptothecin | *CDKN1A* | 10.0 | 7.1 | 2.3 | 2.3 |
| TPA | *CDKN1A* | 1.7 | 2.0 | 1.5 | 1.5 |
| TPA | *EGR1* | 27.8 | 14.2 | 4.5 | 7.1 |
| NaCl | *CDKN1A* | 2.0 | 1.7 | 1.6 | 0.9 |
| Heat shock | *HSPA1L* | 76 | 53 | 13.6 | 16.5 |
| $H_2O_2$ | *CDKN1A* | 4.2 | 10.6 | 1.6 | 2.9 |
| Arsenite | *CDKN1A* | 9.5 | 7.6 | 3.7 | 4.2 |

clonogenic survival is detailed in Supplementary Data Set 1.)

### *Microarray hybridization and general gene expression signatures*

RNA was labeled and hybridized to 7668 element microarrays representing mostly named genes. The resulting gene expression data were filtered for targets with good quality and intensity of hybridization across all experiments (Koch-Paiz *et al*., 2004), and for significant change (increase or decrease) in expression in at least 10% of the experiments. This yielded a set of 1451 genes (Supplementary Data Set 2) that were used in subsequent analyses. Interestingly, when the information from this set of responsive genes was displayed in a multidimensional scaling (MDS) plot, a general grouping of treatments was apparent (Figure 2a). These groups corresponded to the four broad categories of initial action listed in Table 1. Since such a strong apparent general mechanism of action signature was evident across all responsive genes, we next attempted to define a subset of genes that best discriminated between these four treatment classes. Using a gene selection approach combining the results from maximum pairwise t-statistic, distance-based method, class-correlation and Wilcoxon/Kruskal–Wallis Statistic gene selection algorithms, we identified a set of 346 genes that discriminated among these four broad mechanisms of initial action (Gene list in Supplementary Data Set 3). The improvement in classification is illustrated by the MDS plot in Figure 2b.

In order to test the robustness of this set of genes in discriminating general mechanism of stress agent action, TK6 and NH32 were exposed to an additional oxidative stress agent, $CdCl_2$. This new experiment was repeated twice, and RNA was labeled and hybridized to the 7668 element microarrays. When the previous analysis was repeated with the addition of the $CdCl_2$ data, we found that the gene expression response to $CdCl_2$ was most similar to that of sodium arsenite, the other heavy metal used in these experiments. MDS using the previously selected 346-gene signature clearly identified $CdCl_2$ as an oxidative stress agent, and illustrates the similarity of its gene expression response to that of arsenite (Figure 2c).

### *Mechanism-specific gene expression signatures*

The same gene selection methods were applied to the original data set of 1451 genes to identify genes discriminating between treatments causing rapid DNA damage as a primary mechanism of action (UVB, MMS, adriamycin, CPT, cisplatin, $\gamma$-ray, neutron, $H_2O_2$, and arsenite), and nongenotoxic stresses (heat shock, osmotic shock, and TPA). A set of 117 genes discriminated between these general classes of stress treatment (Supplementary Data Set 4), and the relative expression of these genes in response to the various stresses is illustrated in the heatmap in Figure 3a. Figure 3b illustrates an MDS plot of this genotoxic-discriminating signature.

Signatures discriminating individual agents were also identified using the same methods. For instance, 251 genes specifically discriminated arsenite from the other

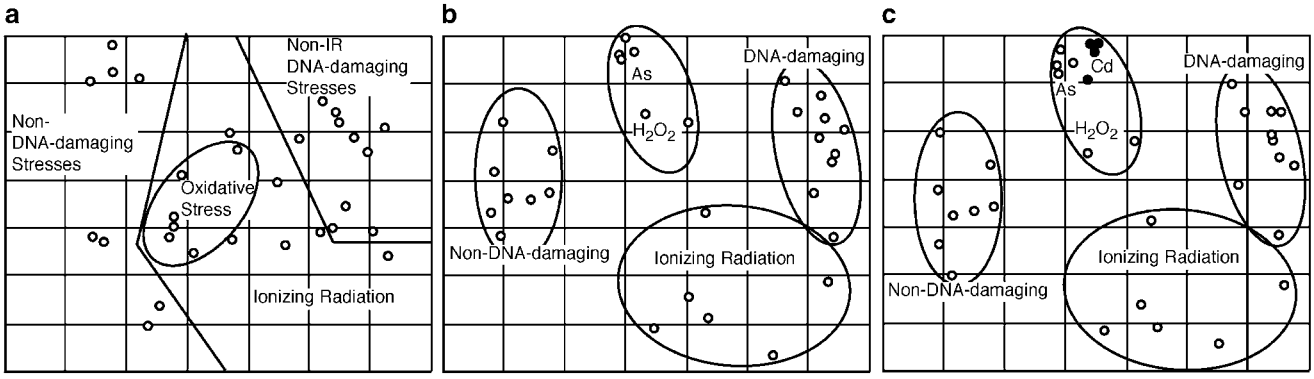


**Figure 2** Two-dimensional projections of multidimensional scaling (MDS) analysis of gene expression in TK6 and NH32 cells following treatments with diverse stress agents. (**a**) MDS using microarray data from all 1451 responsive genes (no selection discriminating between treatments). (**b**) Stress agents were grouped into four broad categories of action: Non-DNA-damaging stresses (heat shock, osmotic shock, and TPA), ionizing radiations (neutron and $\gamma$-ray exposures), other DNA-damaging agents (UVB, MMS. adriamycin, camptothecin, cisplatin), and agents causing mainly oxidative stress (arsenite and $H_2O_2$). Gene selection identified a set of 346 genes that enhanced the separation of the four agent groups. (**c**) TK6 and NH32 were treated with cadmium chloride, and the data from replicate experiments (filled circles) was added to the MDS analysis using the same 346-gene signature identified in Figure 2b

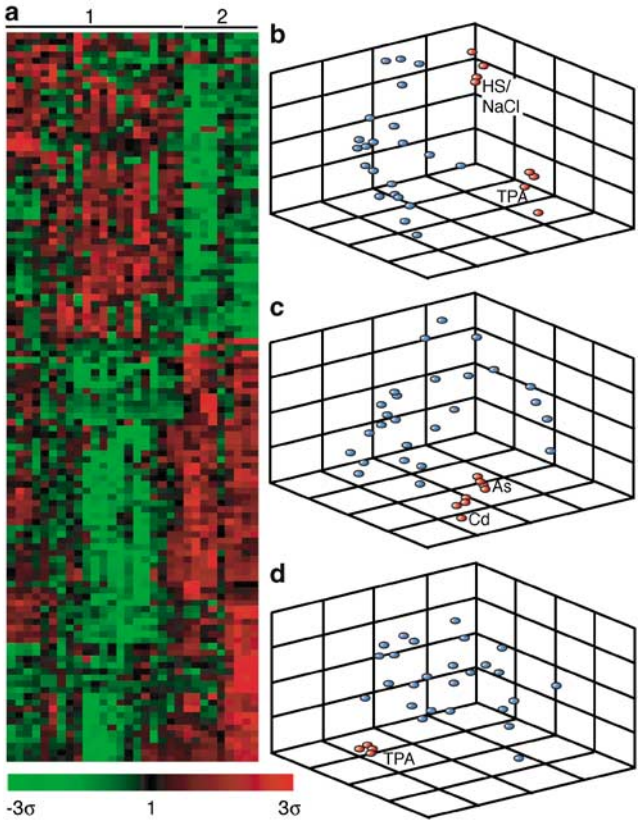


**Figure 3** (**a**) Heatmap of 117 genes (rows) discriminating between genotoxic and nongenotoxic stress. Group 1: Genotoxic treatments. Group 2: Nongenotoxic treatments. Color indicates deviations from the mean expression of each gene following treatment according to the scale below. (**b**) MDS plot of the 117-gene profile from Panel a. Agents not inducing rapid DNA damage (red: TPA, HS (heat shock), NaCl (osmotic shock)); genotoxic agents (blue). (**c**) MDS plot of 251-gene arsenite-discriminating profile. As (arsenite) and Cd (cadmium chloride): red. All other treatments: blue. (**d**) MDS plot of 66-gene profile discriminating TPA (red) from all other treatments (blue)

treatments (Supplementary Data Set 5). Although the $CdCl_2$ data was not used to define this profile, a high degree of similarity in the responses to arsenite and $CdCl_2$ is evident in Figure 3c, where this data is included in the MDS plot using the arsenite-discriminating signature. This suggests a generally robust heavy metal-specific signature. A signature of 66 genes discriminated TPA treatments (Figure 3d, and Supplementary Data Set 6), while only 27 genes were identified that discriminated between ionizing radiation ($\gamma$-ray and neutron treatments) and all the other stresses (Supplementary Data Set 7), possibly indicating a high degree of similarity in the patterns of gene expression response among DNA-damaging treatments.

### *Effects of p53 status in the response to diverse stress agents*

The previous analyses all used information from both the p53 wild-type and p53-null cell lines. As p53 plays an important role in mediating the mammalian stress response to many agents, it was of interest to examine the influence of p53 status on the gene expression profiles obtained in this study. From the complete data set of 1451 genes, we found only 13 genes that discriminated p53 status. However, when the resulting MDS plot was examined, it became evident that while the genotoxic treatments elicited a strong p53-dependent response, the nongenotoxic treatments remained clustered by treatment, and did not show the same p53-dependent pattern as the genotoxic treatments. Further analysis using only the data from the nongenotoxic treatments did not reveal any significantly p53-dependent genes among the responses to the nongenotoxic agents (see Supplementary Data Set 8), indicating that the p53 pathway did not play a major role in any gene expression responses to these stresses. When the analysis of p53 effects was repeated using only

**Table 3** Genes discriminating p53 status after DNA-damaging treatments

| *Gene* | *γ-ray (8 Gy)* | | *MMS (50 μg/ml)* | | *TPA (200 nM)* | |
|---|---|---|---|---|---|---|
| | *TK6* | *NH32* | *TK6* | *NH32* | *TK6* | *NH32* |
| *CDKN1A* | 11.2 | 1.4 | 10.3 | 2.6 | 2.0 | 1.5 |
| *CCNG1*[a] | 1.5 | 0.9 | 1.3 | 0.8 | 1.0 | 1.0 |
| *BAI3* | 4.3 | 1.5 | 2.3 | 1.1 | 1.4 | 1.3 |
| *BTG1* | 2.2 | 1.0 | 1.5 | 1.0 | 2.7 | 2.8 |
| *ST14* | 2.5 | 1.3 | 1.6 | 1.0 | 1.7 | 1.4 |
| *PHLDA3* | 2.8 | 0.9 | 4.9 | 1.4 | 0.8 | 0.8 |
| *PLXNB2* | 2.3 | 1.1 | 2.3 | 1.1 | 1.0 | 0.9 |
| *MDM2*[a] | 4.1 | 1.2 | 3.7 | 1.1 | 1.1 | 1.1 |
| *LIF* | 4.1 | 1.1 | 1.9 | 1.0 | 2.0 | 2.0 |
| *BTG2*[a] | 4.2 | 1.3 | 2.8 | 1.0 | 1.9 | 1.5 |
| *PPM1D* | 2.8 | 1.1 | 2.5 | 1.0 | 1.0 | 1.0 |
| *CTSD* | 2.1 | 0.9 | 1.3 | 0.8 | 0.7 | 0.8 |
| *TRIM22* | 3.5 | 1.0 | 2.0 | 0.8 | 0.8 | 0.9 |
| *XPC* | 3.8 | 1.1 | 2.4 | 1.3 | 0.6 | 0.6 |
| *DDB2*[a] | 2.7 | 1.1 | 1.9 | 1.0 | 0.6 | 0.7 |
| *TNFSF9*[a] | 1.6 | 1.0 | 2.1 | 0.9 | 1.4 | 1.3 |

[a]Genes represented by two spots on the array. Numbers are the mean of all array hybridizations. The genotype of TK6 is *p53+/+* and NH32 is *p53−/−*

the data from the genotoxic treatments, a 16-gene signature (Table 3) was identified that discriminated p53 status broadly across all the genotoxic agents. This signature included all 13 genes originally identified from the complete data set plus *BTG1, BTG2*, and *ST14*. The effect of p53 status on induction of these genes by exemplar genotoxic (γ-ray and MMS) and nongenotoxic (TPA) stresses is illustrated in Table 3.

## Discussion

This study compared the functional genomic responses of human cells to a broad set of stress treatments representing several diverse general mechanisms of cellular damage. The strategy was to compare treatments producing robust gene induction at early times in the absence of pronounced cytotoxicity. This early response after treatment is more likely to reflect the initial response to damage while minimizing secondary responses associated with late cell death. This approach yielded gene expression profiles discriminating between general classes of stressors, and also discriminating individual stresses. The extent to which overall mechanism of action signatures were apparent across the entire unselected set of 1451 responsive genes was quite striking. Such a broad general expression signature has not been reported before, and is in contrast with one study where no general pattern was found for 100 agents compared with an array of 250 reported stress responsive genes (Burczynski *et al*., 2000). It is likely that such a small sampling of genes was not informative for the majority of agents in their very diverse set. By analysing the genes, which did respond in our study rather than those predicted by the literature to respond, it is likely that our gene set included relatively more information content and less noise. The strength of the gene expression patterns we observed across a diverse set of agents supports the hypothesis that gene expression signatures will be distinct enough to be of use in toxicology studies and predicting mechanisms of toxicity.

The mechanistic signatures evident in the data from all the responsive genes also far overwhelmed any effects of p53 genotype. As p53 has been considered one of the major determinants of gene induction patterns following many types of stress, this finding suggests that the mechanism of action of a specific stress agent may be a stronger determinant of gene expression patterns than cellular genotype, or by extension, possibly even interindividual differences. Similar results were reported in a more restricted study comparing γ-irradiation with UV radiation, adriamycin, and cisplatin, where no dependence on p53 status was found within the gene expression signatures (Park *et al*., 2002). This suggests that while p53 strongly regulates a subset of genes in response to specific stresses, the majority of stress-regulated genes are p53-independent. Cell type differences are still likely to play a role in these responses, as has been documented for individual agents, but the strength of the patterns we have found is encouraging for the development of toxicologically informative gene expression databases.

Besides the broad mechanism of action differences evident across all the responsive genes, more specific gene expression signatures were also present within the data. One way of examining these signatures is to compare the Gene Ontology annotations of the genes defining these signatures. The Expression Array Systematic Explorer (EASE) tool allows determination of statistically over-represented Gene Ontology categories within a gene set compared with the frequency among genes on the array (Hosack *et al*., 2003). Thus, this analysis identifies molecular functions and cellular processes represented in a set of genes more frequently than expected by chance alone, indicating possible key mechanisms involved in the process of interest. EASE analysis of our discriminating signatures is summarized in Table 4. Interestingly, the most over-represented molecular function within the DNA-damage discriminating signature was DNA-binding activity, followed by regulation of nucleic acid metabolism and transcription factor activity. In contrast, heat shock, protein folding, and metal ion-specific functions significantly dominated the heavy metal-specific signature. And while the TPA-discriminating signature included genes with many diverse functions, the most significant categories here were phosphatases and inflammation and cytokine responses.

In general, the molecular functions identified as significantly defining the signatures examined were consistent with known modes of action and gene induction patterns. For instance, metallothioneins, heat-shock proteins, and genes involved in transcription have generally been found in microarray profiling studies to be major responders to both arsenite (Hamadeh *et al*., 2002a, b; Rea *et al*., 2003) and cadmium stresses

**Table 4** Gene Ontology categories over-represented within treatment-defining gene expression signatures

| | *EASE score*[a] | *Fisher Exact*[a] |
|---|---|---|
| *TPA* | | |
| Protein-tyrosine-phosphatase activity | 0.00062 | 0.000023 |
| MAP kinase phosphatase activity | 0.0019 | 0.000032 |
| Cytokine activity | 0.0038 | 0.00048 |
| Antiapoptosis | 0.0059 | 0.00052 |
| Response to wounding | 0.0066 | 0.0013 |
| Inflammatory response | 0.0092 | 0.0015 |
| *Heavy metals* | | |
| Heat–shock protein activity | 0.000081 | 0.0000064 |
| Transcription cofactor activity | 0.00067 | 0.00019 |
| Copper ion homeostasis | 0.00072 | 0.000016 |
| Transition metal ion homeostasis | 0.0024 | 0.00011 |
| Protein folding | 0.0026 | 0.00055 |
| Heavy metal sensitivity/resistance | 0.0053 | 0.000079 |
| Copper/cadmium binding | 0.0057 | 0.000088 |
| *DNA damage* | | |
| DNA binding | 0.000032 | 0.000012 |
| Reg. nucleic acid metabolism | 0.000089 | 0.000033 |
| Transcription factor activity | 0.00020 | 0.000054 |
| Nucleic acid binding | 0.0014 | 0.00068 |
| Mitotic cell cycle | 0.038 | 0.009 |

[a]Statistics calculated using the Expression Array Systematic Explorer (Hosack *et al*., 2003)

(Koizumi and Yamada, 2003). It should be noted, however, that only regulation of individual genes from these categories was reported in these prior studies. There was no indication of the relative significance of functional classes in the overall response signature, and the reported gene responses did not distinguish metal stresses from any other mechanism of toxicity.

Even this relatively modest study highlights the enormous richness of information inherent in gene expression responses to stress, yielding general mechanism-of-action signatures, agent-specific signatures, and genotype-specific signatures. When the predictive power of both the general mechanism-of-action signature and the arsenite-specific signature was tested by the introduction of data from a new stress agent, $CdCl_2$, both signatures correctly identified this agent as most like arsenite, indicating the robust nature of the defining gene expression profiles. The functional status of the tumor suppressor gene p53 could also be discriminated by a relatively small set of genes, but only among the DNA-damaging stresses, while no effect of p53 status could be discerned among nongenotoxic treatments. The results of this study provide a solid platform for future mechanistic studies of stress response and molecular toxicology.

## Materials and methods

### *Cell culture and treatment with stress agents*

TK6 (Skopek *et al*., 1978) and NH32 (Chuang *et al*., 1999) were grown in RPMI 1640 medium with 10% fetal calf serum in a humidified, 5% $CO_2$ atmosphere at 37°C. Stock cultures were maintained at $1–12 \times 10^5$ cells/ml, and were tested periodically and found to be mycoplasma-free. Exponentially growing cells were treated at $4–5 \times 10^5$ cells/ml unless otherwise specified. For $\gamma$-ray exposures, cells were irradiated at 2.96 Gy/min to a dose of 0, 2.5, or 8 Gy using a Mark I-68 $^{137}Cs$ source (JL Shepherd and Associates, Inc.). For UVB exposure, cells were pelleted by centrifugation, resuspended in 'RPMI saline' (100 mM NaCl; 5.4 mM KCl; 11 mM d-glucose; 5.6 mM $Na_2HPO_4$; 0.4 mM $MgSO_4$; 0.4 mM $CaCl_2$) and irradiated as previously described (DeLuca *et al*., 1983) with Westinghouse FS20 SunLamp bulbs (270–385 nm emission spectrum with peak at 313 nm) to a dose of 100 J/m$^2$. Mock-irradiated controls were treated identically except for the UV radiation exposure. The cells were then resuspended in fresh medium and incubated at 37°C. Neutron exposures were carried out at the Radiation Research Accelerator Facility (RARAF) of Columbia University. Cells were exposed to 0.43 MeV neutrons in completely filled T-25 flasks in HEPES-buffered RPMI to a total dose of 50 cGy.

All chemicals were obtained from Sigma-Aldrich. Fresh stocks were prepared, added to the culture medium to the concentrations listed in Table 1, and incubated for 4 h, when either the RNA was harvested, or the cells were pelleted by centrifugation and resuspended in fresh medium. Simultaneous controls for each experiment were treated with an equal volume of the diluent (PBS or DMSO) and handled identically to the treated cultures. For osmotic shock treatment, the osmolarity of the medium was adjusted to 500 mOsM by the addition of NaCl for 4 h. Heat-shock treatment was performed by submerging 35 ml cultures in a 47°C circulating water bath with gentle agitation for 20 min, while controls were held in the same volume at 37°C. Time– and dose–response studies were also performed for most agents to determine the dose for the microarray experiments.

At the end of the 4-h treatment period, an aliquot of cells was scored for trypan blue exclusion using a hemacytometer.

### *RNA isolation and quantitative hybridization analysis*

At 4 h after treatment, RNA was extracted using a modified guanidine thiocyanate method (Chomczynski and Sacchi, 1987). RNA for microarray analysis was further purified using RNeasy columns (Qiagen, Valencia, CA, USA). For quantitative measurement of individual genes, serial dilutions of RNA were immobilized on nylon membranes, hybridized with cDNA probes at 55°C in Hybrisol I (Serologicals, Norcross, GA, USA), and washed as described (Koch-Paiz *et al*., 2000). For hybridization probes, cDNA inserts were excised or PCR amplified from the microarray clones, and labeled with $^{32}P$ using random primers (Stratagene, La Jolla, CA, USA). Hybridization was quantified on a phosphorimager (Amersham Biosciences, Piscataway, NJ, USA), and relative signal levels, normalized to the polyA content of each sample, were determined using the RNA-Think program. With this approach, the values for relative RNA are directly proportional to RNA abundance and differences of 1.5-fold or more can be reliably measured (Koch-Paiz *et al*., 2000).

### *Microarray hybridization and analysis*

In total, 100 $\mu$g of whole-cell RNA was labeled and hybridized to 7668 element cDNA microarrays as described previously (Amundson *et al*., 1999). In brief, probes were PCR amplified from IMAGE consortium clones and arrayed on poly-L-lysine-coated glass slides. Fluorescently labeled cDNA was prepared from control and treated TK6 or NH32 whole-cell RNA by a single round of reverse transcription with Superscript II (Invitrogen, Carlsbad, CA, USA) in the presence of fluorescent

dNTP (Cy3 dUTP or Cy5 dUTP, Amersham Biosciences, Piscataway, NJ, USA). Probes and targets were hybridized together for 16 h in $3 \times$ SSC at 65°C in the presence of the blockers human CoT1 DNA, yeast tRNA, and polydeoxyadenine. Hybridized slides were washed at room temperature twice in $0.5 \times$ SSC, 0.01% SDS for 5 min and again in $0.06 \times$ SSC. Cy3 and Cy5 fluorescences were scanned using a laser confocal scanner (Agilent Technologies, Palo Alto, CA, USA), and images were analysed using the ArraySuite 2.1 extensions (Y Chen, National Human Genome Research Institute (NHGRI)) in IPLab (Scanalytics Inc., Fairfax, VA, USA) to calibrate relative ratios and develop confidence intervals for their significance (Chen *et al*., 1997). The ratios were normalized to those of a set of 88 internal controls (DeRisi *et al*., 1996) with a theoretical ratio of 1.0. The variance in the housekeeping set was used to determine the significance of expression changes following treatment, and up or downregulated genes were called at the level of 99% confidence. The ArraySuite Extensions analysis also incorporates four separate metrics of hybridization quality for each target, taking into account the intensity, background, and other characteristics of each spot for both fluorochromes to produce an overall quality assessment for each target on the array (Chen *et al*., 2002).

Gene selection, clustering, and visualization analysis was performed using tools developed by the Division of Computational Bioscience of the Center for Information Technology and the Cancer Genetics Branch of the National Human Genome Research Institute at the National Institutes of Health (http://arrayanalysis.nih.gov). Genes discriminating between different treatment conditions were identified using the maximum pairwise t-statistic, distance-based method, Wilcoxon/Kruskal–Wallis statistic, and class-correlation methods. Genes that had a statistical weight greater than that expected from 10 000 randomized iterations were considered as significantly discriminating the groups. The final discriminating gene sets comprised genes identified as significantly discriminating by at least three of the four selection methods, to produce fairly stringent discriminators for each comparison (Koch-Paiz *et al*., 2004).

**Acknowledgements**

We are grateful to S Marino and G Randers-Pehrson for operation of the Radiological Research Accelerator Facility (RARAF) van de Graaff. RARAF is an NIH supported Resource Center through Grants EB-002033 (NIBIB) and CA-37967 (NCI). We thank the Division of Computational Bioscience of the Center for Information Technology at the National Institutes of Health for providing computational resources for this study. Support from the DOE Low Dose Radiation Program, ER 63308, is gratefully acknowledged.

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)