Final Report for Grant Number DE-FG02-05ER64065 from Office of Science, U. S. Department of Energy

Overview

This is the final report for the project entitled "A functional genomics approach using radiation-induced changes in gene expression to study low dose radiation effects *in vitro* and *in vivo*" which has been supported by the DOE Low Dose Radiation Research Program for approximately 7 years. This has encompassed two sequential awards, ER62683 and then ER63308, at the Gene Response Section in the Center for Cancer Research at the National Cancer Institute. The project was temporarily suspended during the relocation of the Principal Investigator's laboratory to the Dept. of Genetics and Complex Diseases at Harvard School of Public Health at the end of 2004. Remaining support for the final year was transferred to this new site later in 2005 and was assigned the DOE Award Number ER64065. The major aims of this project have been 1) to characterize changes in gene expression in response to low-dose radiation responses; this includes responses in human cells lines, peripheral blood lymphocytes (PBL), and in vivo after human or murine exposures, as well as the effect of dose-rate on gene responses; 2) to characterize changes in gene expression that may be involved in bystander effects; and 3) to characterize responses in transgenic mouse models with relevance to genomic stability. A variety of approaches have been used to study transcriptional events including microarray hybridization (1-4), quantitative single-probe hybridization (1-3, 5) which was developed in this laboratory (6), quantitative RT-PCR (3), and promoter microarray analysis using genomic regulatory motifs (7). Considering the frequent responsiveness of genes encoding cytokines and related signaling proteins that can affect cellular metabolism, initial efforts were initiated to study radiation responses at the metabolomic level and to correlate with radiation-responsive gene expression.

Productivity is summarized below for published and in press manuscripts, as well as a U.S. patent in item 25 of this list. There are several additional publications that will be submitted in 2007 that were supported in part by this program. These future publications include one manuscript on in vivo expression profiling analysis in mouse models, one manuscript on radiation responses in human cell lines, at least one on development of stress signatures in human cells, and three manuscripts on radiation metabolomics.

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4. S. A. AMUNDSON, K. T. DO, AND A. J. FORNACE, JR, Induction of Stress Genes by Low Doses of Gamma Rays. *Radiat Res* **152**, 225-231 (1999).
5. C. A. KOCH-PAIZ, R. MOMENAN, S. A. AMUNDSON, E. LAMOREAUX, AND A. J. FORNACE, JR, Estimation of relative mRNA content by filter hybridization to a polyuridylic probe. *Biotechniques* **29**, 708-714 (2000).
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9. S. A. AMUNDSON, AND A. J. FORNACE, JR, Microarray approaches for analysis of tumor suppressor gene function. *Methods Mol Biol* **223**, 141-154 (2003).
10. S. A. AMUNDSON, A. PATTERSON, K. T. DO, AND A. J. FORNACE, JR, A nucleotide excision repair master-switch: p53 regulated coordinate induction of global genomic repair genes. *Cancer Biol Ther* **1**, 145-149 (2002).
11. S. A. AMUNDSON, AND A. J. FORNACE, JR, Monitoring human radiation exposure by gene expression profiling: Possibilities and Pitfalls. *Health Physics* **85**, 36-42 (2003).
12. S. A. AMUNDSON, AND A. J. FORNACE, JR, Complexity of stress signaling and responses. In *3* (M. Karin, et al., Ed.), pp 179-184. Academic Press, San Diego, CA, 2003.
13. S. A. AMUNDSON, A. LEE, C. KOCH-PAIZ, M. L. BITTNER, P. MELTZER, J. M. TRENT, AND A. J. FORNACE, JR, Differential responses of stress genes to low dose-rate gamma-irradiation. *Mol Cancer Res* **445-52**, 445-452 (2003).
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21. S. A. AMUNDSON, K. T. DO, L. VINIKOOR, C. A. KOCH-PAIZ, M. L. BITTNER, J. M. TRENT, P. MELTZER, AND A. J. FORNACE, JR, Stress-specific signatures: expression profiling of p53 wild-type and -null human cells. *Oncogene* **24**, 4572-4579 (2005).
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25. United States Patent # 7,008,768. Title: "Method for Detecting Radiation Exposure." Inventors: Fornace, Amundson, and Trent. Date: March 7, 2006.

Characterization of radiation responses in human cell lines and primary human cells

Over the course of this project, we have employed molecular biology approaches including cDNA microarray technology, to study cellular responses to radiation injury. In experiments using a human myeloid cell line (ML1), we demonstrated induction of mRNA expression of several stress responsive genes by doses of gamma rays as low as 2 cGy (8). For instance, the dose-response for induction of CIP1/WAF1 and GADD45A appeared to be linear over the range of 2 - 50 cGy, and showed no evidence of a threshold for induction in Fig. 1. Interestingly, we actually have seen a "super-linear" effect at very low doses as exemplified by intersection at greater than one in the left panel; this indicates heighten responsiveness relative to dose at doses less than 2 cGy.

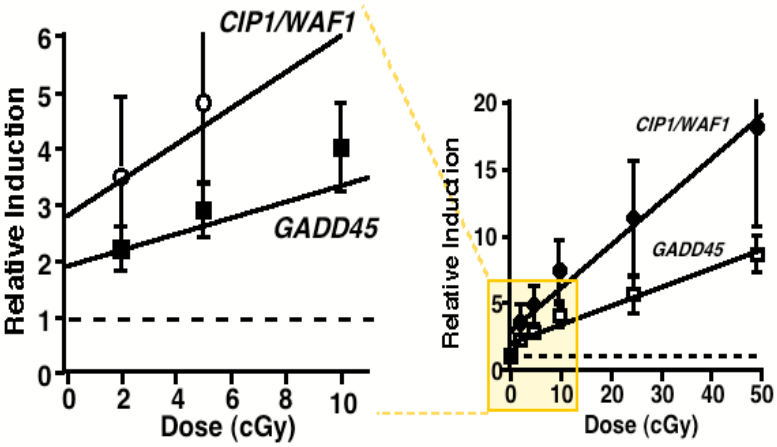

Figure 1. Linear induction of p53-regulated genes in the low dose range. Relative mRNA levels in γ-irradiated ML1 cells is shown relative and is normalized to that of untreated cells, so that no induction would be a value of 1.

Dose-rate effects

Investigation was made into the effects of protracting delivery of low dose ionizing radiation. We found that reducing the dose rate over three orders of magnitude results in some decrease in magnitude, but still causes linear induction of the p53-regulated genes CDKN1A, GADD45A, and MDM2 between 2 and 50 cGy as shown in Fig. 2 (2). Reducing the dose rate reduced the magnitude of induction of CDKN1A and GADD45A (Fig. 2), as well as the extent of apoptosis (Fig. 3). In contrast, MDM2 was induced to the same extent regardless of the rate of dose delivery (Fig. 2) and cell cycle delay also showed a dose-rate-independent effect. Microarray

analysis has identified additional low dose-rate-inducible genes, and indicates the existence of two general classes of low dose-rate responders in ML1 cells.

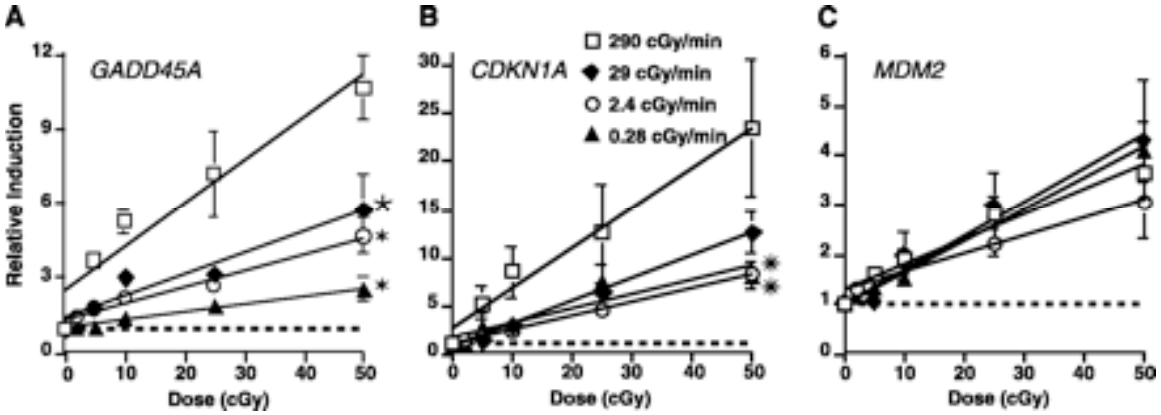

Figure 2. Dose-response relationships for induction of GADD45 (panel A), CDKN1A (panel B), and MDM2 (panel C) by γ-rays delivered at 290 cGy/min, 29 cGy/min, 2.4 cGy/min, and 0.28 cGy/min.

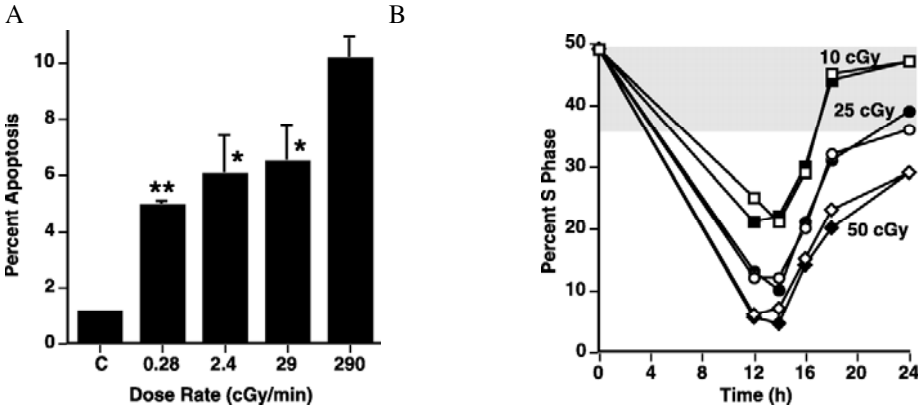

Figure 3. Dose rate effect on cell fate. (A) Percentage of ML1 cells scored as apoptotic by 4',6-diamidino-2-phenylindole staining 48 h after completion of treatment with 0.5 Gy γ-rays delivered at different dose rates. C indicates the level of apoptosis in untreated control cultures. (B) Effect on ML1 cell cycle progression of irradiation at low dose rate. The percentage of cells in S phase is plotted at various times following the conclusion of low dose irradiation delivered at either 2.9 Gy/min (filled symbols) or 0.0028 Gy/min (open symbols). The shaded area indicates the normal range of S phase observed in untreated cells through the course of the experiment.

While the results indicate a generally protective effect of low dose-rate exposures on gene induction, some exceptions were also noted. The differential responses of groups of genes to reduced dose-rate exposure may indicate important differences in the regulatory mechanisms

governing their expression (2), and representative results are shown in Table 1. Interestingly, apoptosis showed a dose rate effect while checkpoint activation did not, and there was some correlation with the spectrum of genes in each set of Table 1.

Gene name	Apoptosis	Cell cycle / Proliferation
Dose-rate Responsive Genes		
CDKN1A		+
Killer/DR5	+	
TRID	+	
Apoptosis (APO-1) antigen 1	+	
Homo sapiens TRAIL receptor 2	+	
Homo sapiens TRAIL receptor 2	+	
TNFSF9	+	
Dose-rate Independent Genes		
BTG2		+
MDM2		+
ELK4		+
MDM2		+
Serum-inducible kinase SNK		+
PPM1D		+
TNFRSF6	+	

Table 1. Comparison of genes showing dose-rate dependence to those showing similar responses regardless of dose rate. Representative results are shown.

Responses in non-growing primary cells

As a first step to study in vivo studies in humans, the responses of peripheral blood lymphocytes irradiated ex vivo were characterized. These experiments used freshly collected blood from donors at the NIH blood bank, from which we separated the lymphocytes using a standard

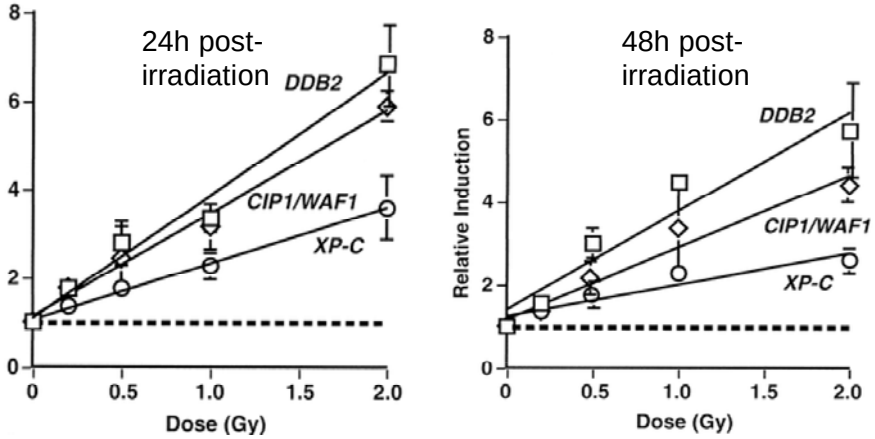

Figure 4. Dose response for the induction of DDB2, Cdkn1a (Cip1/WAf1), and XPC in ex vivo-irradiated human peripheral blood lymphocytes

density gradient centrifugation. We used microarray analysis to identify radiation-regulated genes that could potentially serve as informative biomarkers of radiation exposure. Our initial studies identified several genes significantly up-regulated in human peripheral blood between 24

and 72 hours after *ex vivo* irradiation. Three of these genes, *DDB2*, *CDKN1A* (CIP1/WAF1), and *XPC*, were induced in a linear fashion between 0.2 and 2 Gy at 24 and 48 hours after treatment (Fig. 4), with less linearity at earlier or later times (9). We have extended these studies with murine and human microarrays to examine the *in vivo* responses to whole body irradiation.

Search for low dose specific radiation responses

As already published by my group (1, 2, 8, 9), the magnitude of induction for most radiation-responsive genes has been found to be approximately proportional to dose – particularly in the 2 to 200 cGy range. We have observed this both in human cell lines as well as in primary cells such as human peripheral white blood cells (WBC). Considering the programmatic emphasis on low dose responses, substantial effort has been made to identify responses occurring at low doses in the 10 cGy range. Initial efforts have focused on p53 wt human cell lines. Interestingly, we have found a subset of stress genes that show appreciable responsiveness, as measured by increased mRNA levels, at low dose but with much less responsiveness at 2 Gy.

Human *in vivo* radiation-induced biomarkers:

In collaboration with Dr. Joel Greenberger at the Univ. of Pittsburgh and Dr. Marcy Grace A.F.R.R.I., we have determined gene expression changes that are triggered in vivo in WBC. This work builds upon our results using quiescent WBC in short-term culture and ex vivo irradiation (9). Blood was drawn from patients immediately prior to and 6 hours following fractions of 1.5 Gy total body irradiation in preparation for bone marrow transplant. We first identified *in vivo* radiation-responsive genes by microarray analysis. Although the general *in vivo* patterns of stress-gene induction appear similar to those obtained from *ex vivo* WBC experiments, additional radiation-responsive genes have been identified. While relatively few genes were down regulated following the initial radiation fraction, a strong down-regulation response was apparent after the second fraction. In contrast to our prior findings with irradiated cell lines, there is not a significant involvement of a large number of cell cycle regulatory genes among *in vivo* radiation-repressed genes. This may be a result of the quiescent nature of the unstimulated WBC used in this study in contrast to the transformed cell lines used in prior studies. Instead, the predominant functional gene ontology classification of both up- and down-regulated genes identified in this *in vivo* study identified many genes associated with inflammatory responses as well as heat shock responses. Results for these and other in vivo experiments are summarized in Table 2. To quantitatively measure single gene induction in samples containing limited amounts of RNA, we switched from our standard approach (6) to a RT-PCR approach in collaboration with Dr. Grace. A progressive increase in mRNA levels relative to unirradiated controls was observed for several radiation-inducible genes. Interestingly, some inter-individual variability in the magnitude of the responses was seen when samples from different patients were analyzed with this RT-PCR approach. The observed variations in overall individual responses substantiates our earlier prediction that individual genes would not have great value as either exposure or diagnostic biomarkers, but that the overall expression pattern of a set of genes would likely be more informative. This *in vivo* validation marks an important step in the development of potentially informative radiation exposure biomarkers for use in monitoring of exposed individuals.

Radiation responses in a panel of human cell lines.

The 60 cell lines of the NCI Anti-Cancer Drug Screen (NCI60) constitute the most extensively characterized *in vitro* cancer cell model, and have been tested for sensitivity to over 100,000 potential chemotherapy agents (10, 11). We have used the NCI60 cell lines and three additional lymphoblast lines to develop a database of responses of different human cell lines to ionizing radiation. We compared clonogenic survival, apoptosis induction, and gene expression response by microarray analysis. While several studies have measured relative basal gene expression in

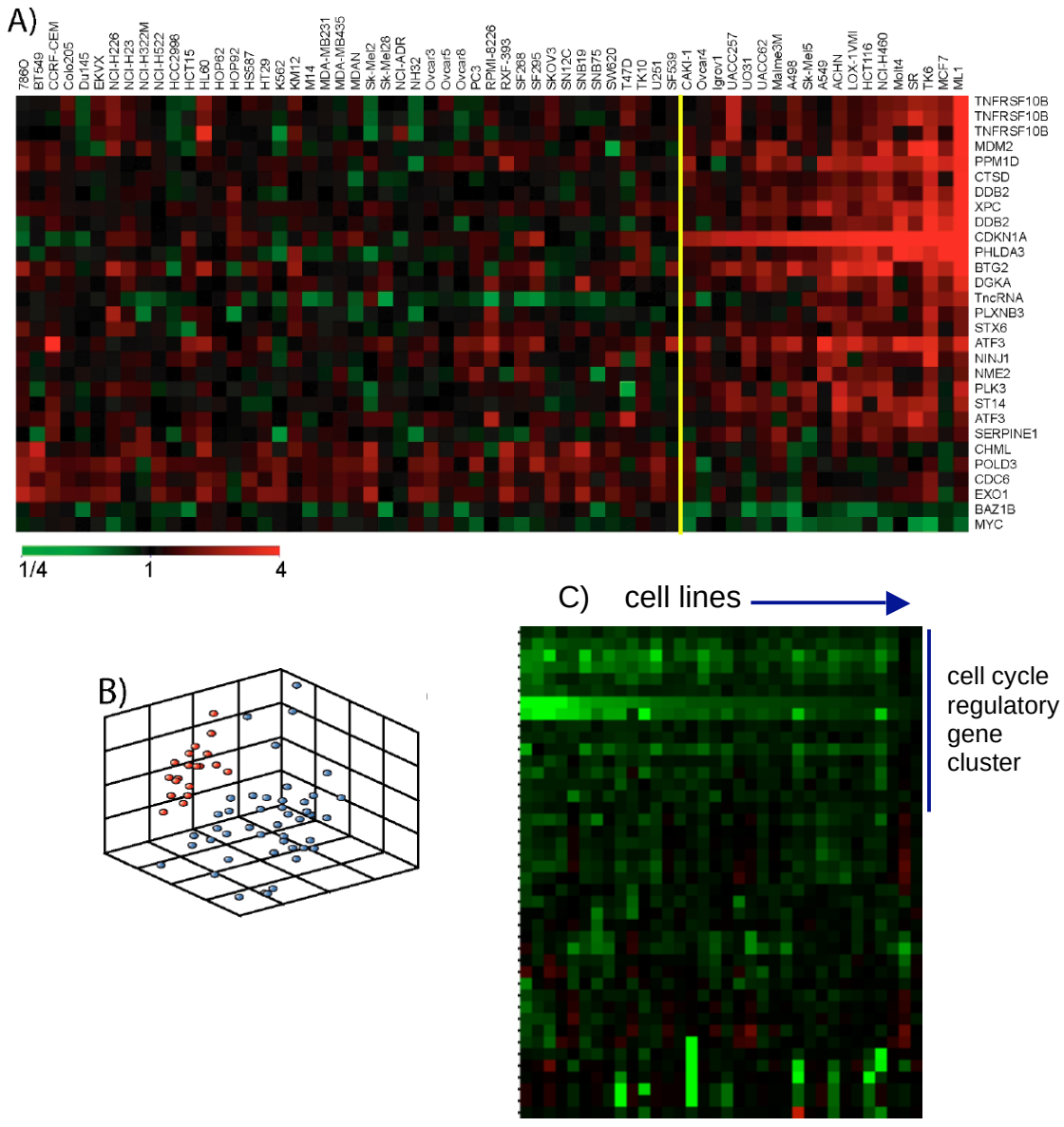

Figure 5. p53 status and radiation responsive genes. A) Heat map showing fold-change ratios in each cell line of the 25 genes identified as discriminating p53 status. Cell lines with mutant p53 are to the left of the yellow line, p53 wild-type lines to the right. B) MDS plot showing separation of p53 wild-type (red) and p53 mutant (blue) cell lines by expression of these same 25 genes. C) Heatmap of fold-change ratios for genes showed a widespread down-regulation. Blue bar marks cluster of genes that involve in cell cycle regulation.

the NCI60, this is the first comparison of large-scale gene expression changes in response to genotoxic stress. We found a set of 22 genes to be differentially regulated in cells with low survival after 2 Gy γ-rays, while 14 genes similarly discriminated the lines sensitive to 8 Gy. In contrast to reported basal gene expression patterns, little tissue-of-origin effect was detected in the radiation response pattern of gene expression, with the exception of lymphoblastoid cell lines. Basal expression patterns, however, discriminated well between radiosensitive and more resistant cell lines, possibly being more informative than the radiation response signatures. The most striking patterns in the radiation data were a set of genes upregulated preferentially in the p53 wild-type lines (Fig. 5A and B), and a set of cell-cycle regulatory genes strongly down-regulated across the entire NCI60 panel (Fig. 5C). The response of these genes to γ-rays appears to be unaffected by the myriad of genetic differences across this very diverse cell set, and represents the most universal gene expression response to ionizing radiation yet observed.

Development of genotoxic stress-specific signatures

Efforts have been made to define patterns of responses to various stresses. To use gene expression as a monitor for radiation exposure, development of radiation-specific response profiles is needed to distinguish from other injury responses. As a first step, we have examined gene expression responses of human cell lines exposed to a diverse set of stress agents. The B-lymphoblastoid cell line TK6 (p53 wild-type) and its p53-null derivative, NH32, were treated in parallel to facilitate elucidation of p53-dependent responses. RNA was extracted four hours 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 (TPA)], agents causing mainly oxidative stress (arsenite, cadmium, and H_2O_2), 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 dataset, 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 (4). A set of 16 genes did exhibit a robust p53-dependent pattern of induction in response to all nine DNA-damaging agents, however. A MDS plot is shown in Fig. 6A to highlight the differences in general responses between these four subsets of stress agents using 346 selected genes; these subsets could be distinguished even with an unselected set of over 1,000 genes (4). Results in Fig. 6B employed full-genome microarrays and highlight the clear difference between genotoxic and non-genotoxic agents.

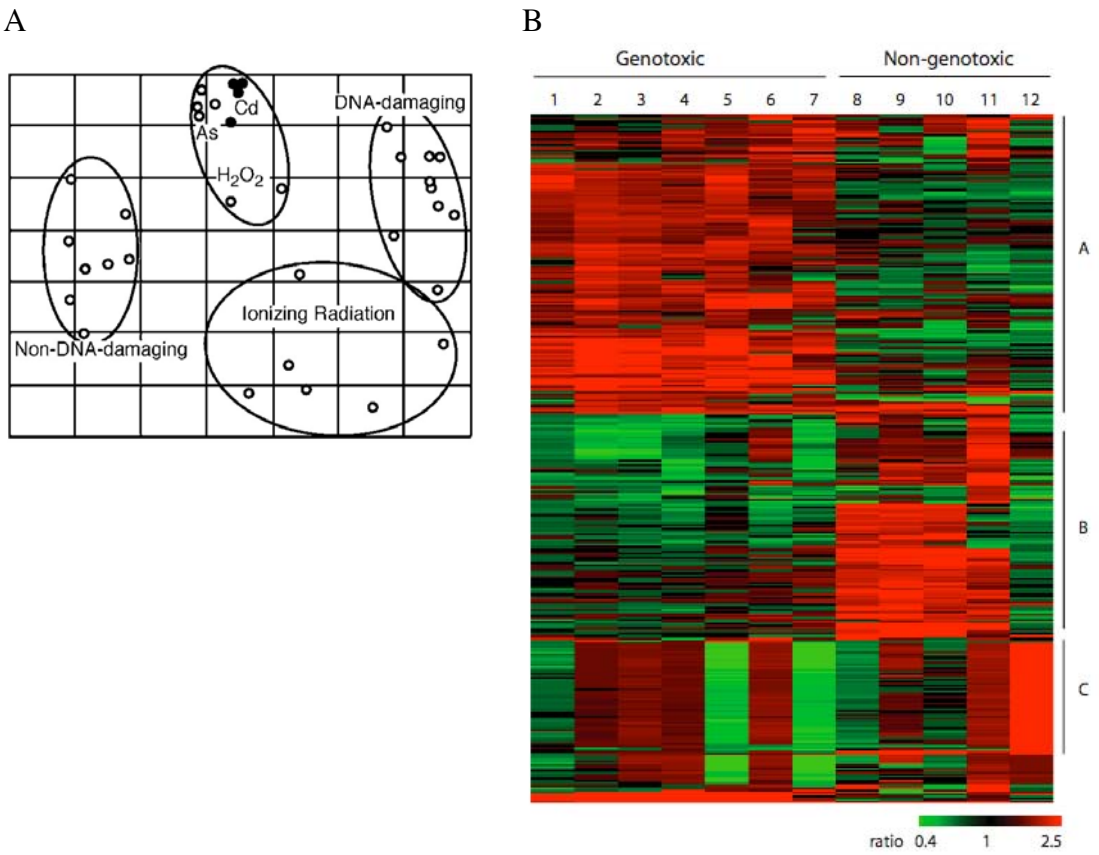

Figure 6 (A) Example of similarities in overall gene expression signatures for agents with similar mechanisms of action. Two-dimensional projections of multidimensional scaling (MDS) analysis of gene expression in p53 wt cells (TK6) and its p53-null derivative (NH32) are shown for treatments with diverse stress agents. Stress agents were grouped into four broad categories of action: Non-DNA-damaging stresses (heat shock, osmotic shock, and TPA), ionizing radiations (neutron and γ-ray exposures), other DNA-damaging agents (UV radiation, MMS, adriamycin, camptothecin, cisplatin), and agents causing mainly oxidative stress (arsenite and H2O2). Gene selection identified a set of 346 genes that enhanced the separation of the four agent groups; cells were subsequently treated with cadmium chloride, and the data (filled circles) was added to the MDS analysis using the same 346-gene signature (4). (B) Representative 2-dimensional clustering of 714 genes (rows) that distinguish genotoxic from non-genotoxic type stresses. Genes in group A highlight genes showing more responsiveness for genotoxic stresses, group B show more responsiveness for non-genotoxic stresses, and group C highlight genes prominent responsiveness for heat shock. From left to right, the 12 treatments are 1) x rays, 2) Ara C, 3) hydroxyurea, 4) camptothecin, 5) bleomycin, 6) hydrogen peroxide, 7) cisplatin, 8) thapsgargin, 9) tunicamycin, 10) 2-deoxyglucose, 11) antimycin, and 12) heat shock. TK6 ells were treated with doses showing robust responses and harvested 4 h after the start of treatment. RNAs were extracted, labeled and hybridized on Agilent human whole genome 44K oligo microarrays that contain more than 41,000 unique human genes and transcripts. Featured Extraction vers. 9.1 was used to filter, normalize, and calculate the signal intensity and ratios. The result files of different treatments were loaded on Rosetta Resolver for 2-dimensional clustering. Treatments and genes were clustered with agglomerative algorithm. Genes with a fold change no less than 1.5 and p value no more than 0.01 in at least three treatments were selected. The color indicates the ratio of the gene signal intensity of treated cells to untreated controls.

Exploration of radiation-activated signaling pathways using mouse models

In addition to genes with roles in cell cycle, apoptosis, and DNA repair, a surprisingly large number of responsive genes were consistently found that encoded cytokines, cytokine receptors and cytokine associated proteins, cell junction and cell membrane proteins, secretory and plasma proteins, acute phase response-related proteins, tissue proteases, and inflammatory mediators (Table 2). A variety of tissues from irradiated mice showed responsiveness for many of these genes. At doses of 150 to 400 cGy, strong acute (4 h) responses were observed that persisted in many cases beyond 48 h. Responses generally showed dose dependence and could be detected, albeit at lower magnitude, to 10 cGy, the lowest dose employed.

Since p53 plays a prominent role in radiation responses in cell lines, ex vivo, and in vivo WBC, we employed Trp53-/- mice to study the role of p53 after irradiation in mouse tissues. Trp53-/- splenic tissue exhibited dramatically attenuated responses in that the number of genes showing significant induction was only half of that of wild type (wt), and many of the remaining responses were of lower magnitude than that of wt. The effect of Trp53-/- on radiation-repressed genes was not as remarkable as that for induced genes. Some representative results are shown in Fig. 7 and 8. We have generated a new mouse strain, carrying a human p53 transgene in the Trp53-/- background. Human and mouse p53 proteins share 77% similarity but there are important differences in some regulatory regions. Expression of human p53 in the Trp53-/- background fails to restore the γ-ray-induced apoptotic response, and does not prevent accelerated tumor development after γ-radiation (12). However these mice do not show early onset of spontaneous tumors as typically seen for Trp53-/- mice. Quantitative single-probe hybridization and RT-PCR were used to determine the relative mRNA levels for selected p53 target genes. The results indicate that human p53 is deficient in transactivating target genes in this model. Further study revealed an unexpectedly high binding of Mdm2 and transgenic p53.

To dissect regulatory components for *in vivo* radiation responses, studies have been carried out in spleen and other tissues in the p38αDN knock-in and Wip1-/- mouse models. Since p38 MAP kinase has a prominent role in mediating inflammatory responses and can phosphorylate sites in p53 which are required for activation, we generated a p38aDN (knockin) mutant where the activating phosphorylation sites at T180 and Y182 were mutated; this mutant p38 (designated p38+/DN) functions as a dominant-negative and markedly reduced p38 signaling after stresses, such as UV radiation (13). p38 can be inactivated by the Wip1 phosphatase, which is known to have oncogenic properties, and Wip1 has recently been found to also dephosphorylate a key phospho-serine in ATM (14). We and others have observed that Wip1-/- cells and mice show heightened p38 activity (15). The p38+/DN model showed reduced induction of some known p53-regulated genes such as Cyclin G, p21 (Cdkn1a), Bax and Apaf1 (Fig. 8). We observed striking ablation of the up-regulation of inflammation-related genes in p38+/DN including a variety of interleukins and other cytokines, as well as genes encoding VCAM1 and matrix metalloproteinases, and enzymes involved in leukotriene biosynthesis, such as arachidonate 5-lipoxygenase. Interestingly, the Wip1-/- model showed the opposite effect with enhanced induction of a variety of cytokines, growth factors, and known p53-regulated genes (Fig. 8). These results highlight the overlap between radiation and inflammatory responses, and demonstrate prominent roles for p38 signaling in radiation signaling *in vivo.*

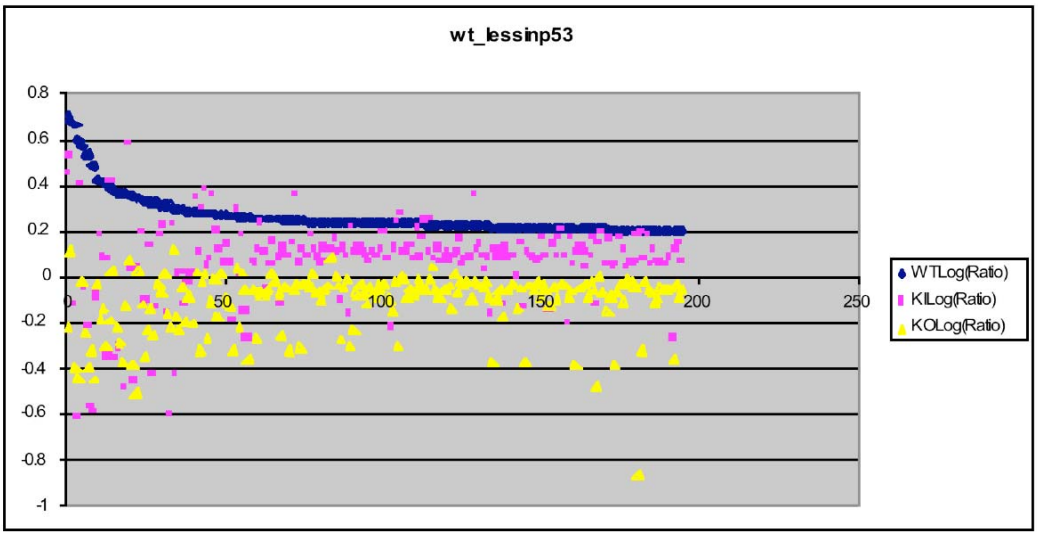

Figure 7. Wild type, p53-null, and p38a+/DN mice were irradiated with 3 Gy. Tissues were harvested at 4 hours after irradiation and RNAs were extracted. We performed microarray on spleen samples. Values of log Ratio (IR/mock) of different mouse strains were plotted. We selected the gene set that showed p53-dependent induction, i.e. genes that had an abolished (or substantially reduced) responsiveness in p53-null mice. Responses were then compared to those in the mice expressing dominant-negative p38α knockin mutant; many genes showed some attenuation in the latter mutant. Legend: blue, responsiveness in wt mice; light red, responsiveness in p53-null; yellow, same in p38a+/DN.

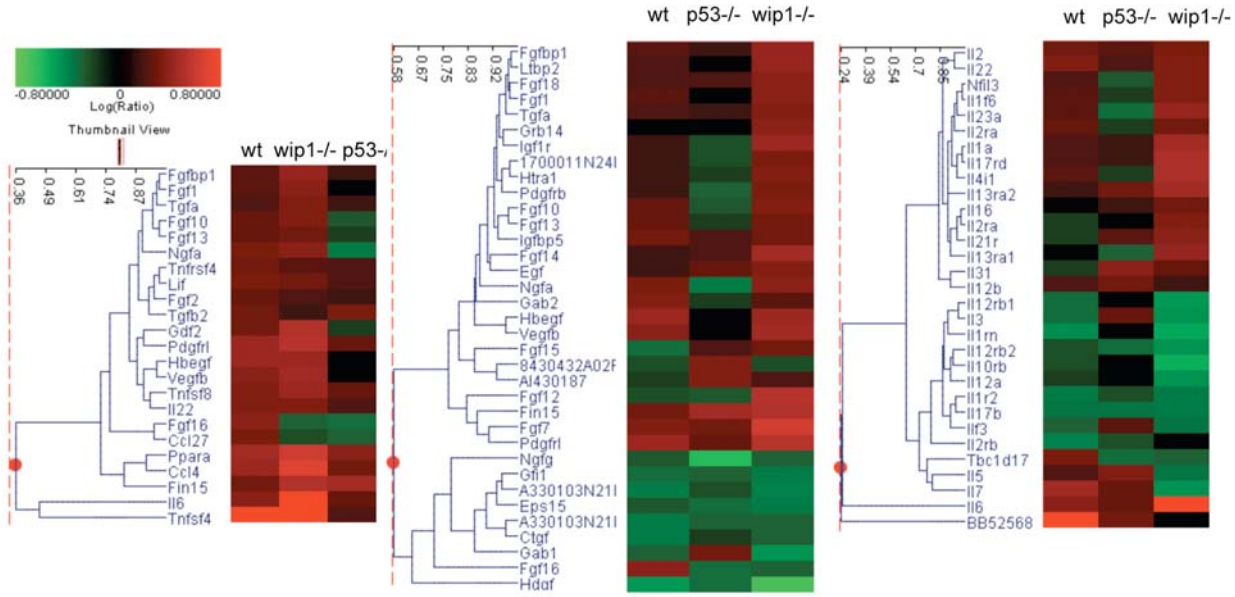

Figure 8. Clustering analyses of microarray data from γ-ray irradiated (4 Gy) mouse spleen harvested at 4 hours after irradiation. Results for genes encoding cytokine and growth factors are shown in the left panel to highlight heightened responses for Wip1-/- compared to wt and reduction in p53-/-. The middle and right panel are clustering of these three lines for growth factors and cytokines respectively. Color thermometer range is from 6.3-fold repression (green) to 6.3-fold induction (red); greater values are indicated by the same color as the maximum.

Table 2. Radiation-inducible genes with roles in intercellular communication, tissue responses, or systemic effects[1]

Cytokines TGFß, TGFß 3, IL-1a, IL-1ß, IL-4, IL-6, IL-15, IL-18, adipsin, calpamodulin, cardiotrophin 1, cholecystokinin, colony stimulating factor 1, cystatin B, epiregulin, Fas antigen, fibroblast growth factor 7, glial cell line derived neurotrophic factor, insulin-like growth factor 1, Leukemia inhibitory factor, lipocalin 2, lipocortin 1, mast cell growth factor, natriuretic peptide receptor 1, neutrophilic granule protein, placental growth factor, prosaposin, prothymosin ß 4, Small inducible cytokines (A3, A4, A6, A9, A11, A12, B9, B11,), thrombospondins (1, 3, 4), TNF receptor superfamily 1b, TNF superfamily member 4, TNF superfamily member 8, TNF superfamily member 10, TRAIL ligand

Receptors and cytokine associated IL-6 receptor α*, IL1 receptor-associated kinase, activin A receptor, type 1, activin receptor IIB, adrenergic receptor ß2, bFGF receptor, calcium modulating ligand, Calcium-sensing receptor, Cannabinoid receptor 1, CD30L receptor, CD36 antigen (collagen type I receptor), CD86 antigen, chemokine receptor 1 tetratricopeptide repeats 2, Chemokine receptor-like 1, cholecystokinin, colony stimulating factor 1 receptor, colony stimulating factor 3 receptor, cytokine inducible SH2-containing protein, EGF receptor pathway substrate 15, Eph receptor A2, Eph receptor A4, ERBB-2 receptor protein-tyrosine kinase, erythropoietin receptor, Estrogen receptor, FGF receptor 4, fibronectin receptor ß, GABA A receptor, GDNF family receptor α 2, Glutamate receptor, metabotropic 3, Human leukemia virus receptor 1 (GLVR1), insulin-like growth factor binding proteins (1, 4, 5, 7), insulin receptor substrate 4, interferon α-inducible protein 27, interferon γ receptor 2, Interferon γ receptor ß chain, interferon γ-induced protein 10, interferon-induced proteins (35, 41, other), killer cell lectin-like receptor subfamily A member 5, killer cell lectin-like receptor subfamily C member 2, LDL receptor related, leptin receptor, leukocyte immunoglobulin-like receptor, melanocortin 2 receptor, natriuretic peptide receptor 1, oxytocin receptor, PDGF receptor ß, peroxisome proliferator activated receptor α, peroxisome proliferator activator receptor γ, platelet-activating factor receptor, protein tyr phosphatase receptor type, receptor 4-1BB ligand, retinoic acid receptor α, retinoic acid receptor, γ, retinoid X receptor interacting protein 110, Retnla, TGFß 1 induced transcript 1, Thyroid hormone receptor α, TRIP9, TNF receptor 5, TPA urokinase receptor, TRAIL receptor 2, Transferrin receptor, B lymphocyte chemoattractant BLC, granulin, chemokine (C-C) receptor 1-like 1

Cell junction and cell membrane gap junction membrane channel protein ß2, gap junction protein ß1 (connexin 32, Charcot-Marie-Tooth), cardiac gap junction protein, calcium-activated potassium channel (SKCA3), voltage-dependent anion channel 3, tight junction protein 1, integrin α E, laminin α 1, vascular cell adhesion molecule 1, thrombomodulin, thrombospondin 4, integrin ß4, integrin ß8, integrin α V, integrin α8, neural cell adhesion molecule, P glycoprotein 1, neural cell adhesion molecule (CALL), selectin L (lymphocyte adhesion molecule 1), syndecan 4, annexin A2, Angiomotin, Cadherin 7 type 2, Cadherin 10, protocadherin gamma subfamily A 4, Intercellular adhesion molecule (Icam1),

Secretory and plasma proteins lactotransferrin, cathelin-like protein, chromogranin B, pentaxin-related gene, fibronectin, uteroglobin, transcobalamin 2, ceruloplasmin, apolipoprotein B, chromogranin A, plasminogen activator inhibitor, type I, plasminogen activator inhibitor type II

Acute phase response-related amyloid ß (A4) precursor, tissue plasminogen activator (TPA), cytochrome P450 2e1, pentaxin-related gene, complement component 2, complement component 3, complement component 1, complement factor H, cytochrome P450 2c29, ferritin light chain 1, fibrinogen-like protein 2, plasminogen, fibrinogen ß, complement component 7, cytochrome P450 reductase, cytochrome P450 51, immediate early response 3 (Ier3), Glutathione S-transferase mu 4

Tissue protease related matrix metalloproteinase 2, matrix metalloproteinase-9, a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 5, ADAM 5 protein precursor (ADAM 5), cathepsin B, plasmin inhibitor α2, and above

Eicosanoid biosynthesis Prostaglandin-endoperoxide synthase 2 (COX2, Ptgs2), Arachidonate lipoxygenase, phospholipase A2

[1] Representative radiation-responsive genes are shown for murine and human (3) in vivo studies; this includes recent results with Agilent whole genome microarray chips. Over 40 of these genes showed attenuated responsiveness in p38+/DN mice.

Radiation Metabolomics

Considering the myriad of radiation-responsive genes that are involved in intercellular signaling and considering that many of the products of these genes, such as cytokines, can have a wide variety of effects on cellular metabolism, we have initiated studies to assess the impact of radiation on metabolism. Our approach utilizes metabolomics, defined as the global analysis of low-molecular weight molecules for identifying context dependent metabolic phenotypes that vary temporally and according to physiological, developmental, or pathological state. We collected 24-hr urine samples from adult male mice before and after single exposure to 10, 300, or 600 cGy. Urines were analyzed by ultra-performance liquid chromatography-time of flight mass spectrometry (MSTOF). Data were analyzed with principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) to identify markers specifically elevated in post-exposure urines compared with pre-exposure and control urines using approaches developed by our collaborator in this project, Dr. Gonzalez and his laboratory at NCI (16-19). Putative chemical formulae for candidate markers were derived based on MSTOF accurate mass measurements. Identities were verified against known standards by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results from PCA and PLS-DA comparing urines from exposed and unexposed mice indicate that urine metabolic profiles change following radiation exposure. While changes in response to 10 cGy irradiation are subtle, we found several metabolites clearly elevated in urines from animals exposed to 3 and 6 Gy compared with pre-exposure and controls. A multi-institutional effort is underway to develop metabolic profiles for radiation biodosimetry at higher doses (1 to 10 Gy); see http://cmcr.columbia.edu/project3.html. The same approach will probably also have utility in the elucidation of metabolic changes induced at low doses of radiation. Representative results are summarized in Fig. 9. Metabolomic studies have also been initiated in TK6 cells in cooperation with Dr. Gonzalez and Dr. Idle (Univ. of Bern). This approach relies on UPLC-MS (TOF) instrumentation at NCI. Preliminary results from γ-irradiated cells

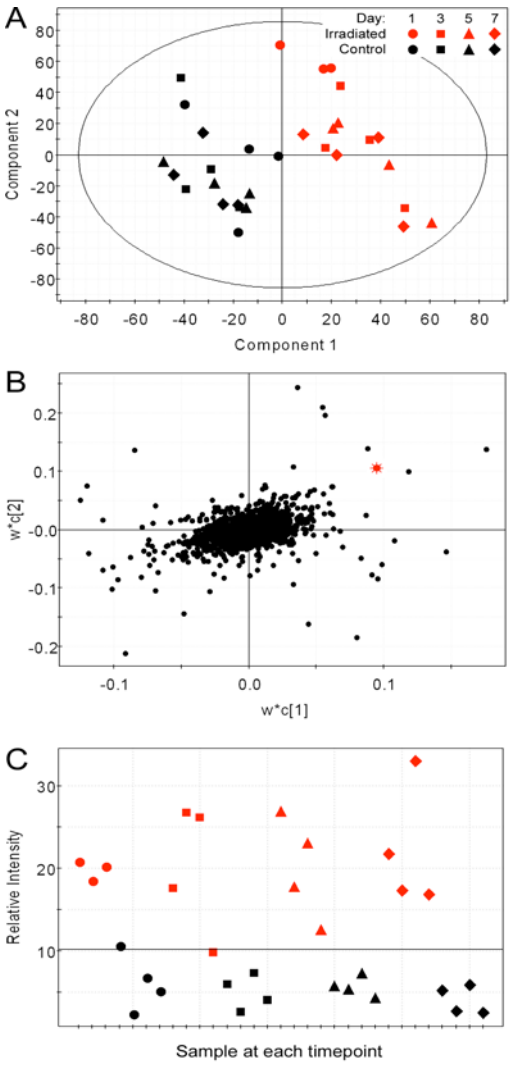

Figure 9 Partial least squares–discriminant analysis (PLS-DA) of mass spectrometry data from urine samples at four timepoints post-exposure of mice exposed to 0 (control, black symbols) or 3 (IR, red symbols) Gy γ-radiation. PLS-DA scores (A) show clear separation of samples according to exposure status. Scores separation is based on individual loadings (ions, B) and how they differ in relative intensity with respect to exposure status. A plot of timepoint-specific relative intensities for a representative individual loading (✳ in B) that is elevated in irradiated versus control animal urine samples is shown (C). The median relative intensity of 10.3 is indicated in C by a horizontal line.

indicate that a good separation by unsupervised PCA analysis can be discerned between irradiated cells and control cells.

Development of a promotor microarray approach to study radiation responses

An important direction for our radiation-responsive gene project is to extend our nucleic acids studies to the proteomics levels. In this regard, our collaborator Dr. Charles Vinson at NCI has developed a state-of-the-art promoter microarray chip. With this approach, we have the capability to survey binding of key transcription factors to regulatory elements in thousands of different mammalian gene promoters. With a supplement to this DOE grant, studies have been initiated using a murine promotor microarray platform. This approach allows us to measure binding of stress-responsive transcription factors to thousands of different regulatory elements both in cell lines and in vivo with various mouse models. A longer term plan is to apply this approach to human cell lines and WBC from irradiated patients. A focus of this project is to identify genome wide changes in DNA binding of stress responsive transcription factor following a stress in new-born primary mouse skin keratinocytes. We have been doing chromatin immunoprecipitation (ChIP) assays with antibodies against several transcription factors that are responsive to stress signals. We take cells and crosslink proteins to DNA using formaldyhde, isolate DNA from the ChIPs and determine the genomic localization of these proteins using the NimbleGen mouse promoter microarrays. These microarrays contain 400,000 oligonucleotides that are each 50 base pairs long that interrogate 26,000 promoters. Each promoter is represented by ~ 15 oligonucleotides that span from -1,000 bp to +500 bp. Fig. 10 shows the results of hybridizing DNA isolated following a ChIP using antibodies against phosphorylated c-Jun following UV treatment. One observes that certain sequence are enriched in the axis of the ChIP DNA while no DNA sequences are enriched in the genomic DNA axis. This indicates that the DNA isolated from the phosphorylated c-Jun ChIP is enriched for certain DNA sequences which we interpret indicates that phosphorylated c-Jun is bound to those DNA sequences in vivo.

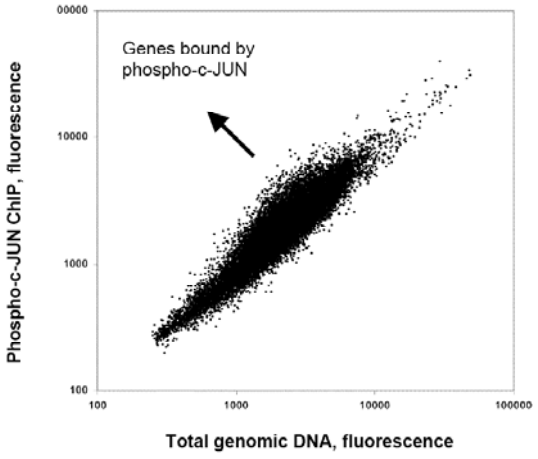

Figure 10 Scattering plot of promoter microarray data, x-axis represents the hybridization intensity of promoters with total genomic DNA and y-axis represent the intensity with DNA immunoprecipitated by phospho-c-JUN. The spots above the diagonal are genes enriched by phospho-c-JUN binding.

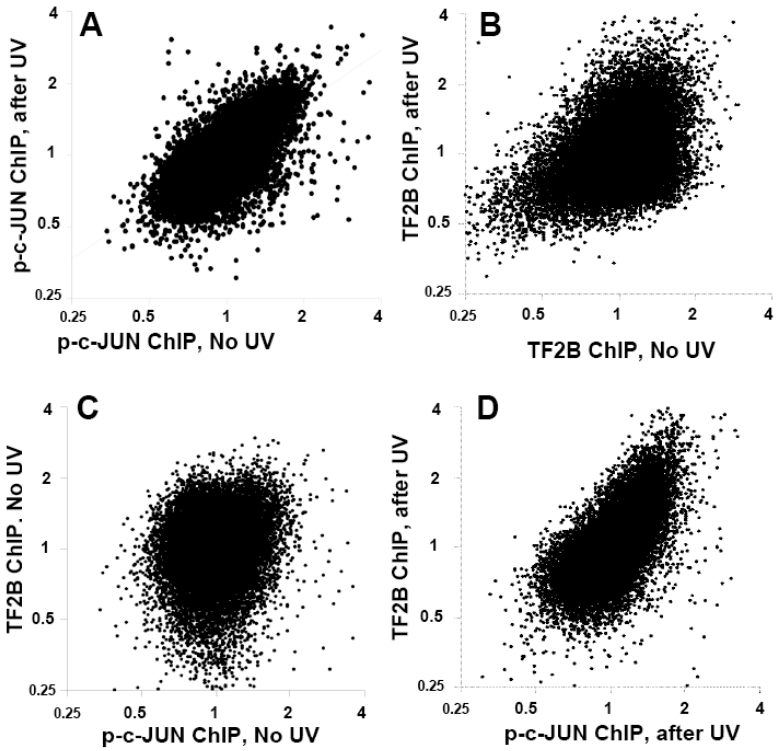

Figure 11. Promoter microarray analysis of cells under normal condition and after UV treatment by chromatin IP with phospho-c-JUN and TF2B.

To evaluate if the localization of a protein changes after an experimental intervention, we determine the ratio of DNA enrichment for a particular promoter in each of the two conditions by doing ChIP in both conditions and plot these results. If there is no change, we produce a diagonal line. The localization of phosphorylated c-Jun in the basal state and 3 hour following UV irradiation is similar (Figure 11A). In our experimental situation, c-Jun phosphorylation increases following UV irradiation but some does exist in the basal case. The most dramatic change in genomic localization we observe occurs for the basal transcription factor TF2B. TF2B is bound to many promoters before irradiation but following irradiation, TF2B is bound to many fewer promoters (Figure 11B). The exciting aspect of this work is when we compare binding of TF2B and phosphorylated c-Jun to the same promoters. Figure 11C shows the binding of these two proteins before irradiation. There is a huge smear suggesting that these two proteins are binding independently of each other. However, following irradiation, there is a diagonal line indicating that they are binding to the same promotes (Figure 11D). Since the genomic localization of phosphorylated c-Jun does not change following treatment, this suggests that the restriction of TF2B localization following treatment is to the proteins bound by phosphorylated c-Jun. We are engaged in trying to understand the interaction between phosphorylated c-Jun and TF2B to mechanistically explain this restricted binding of TF2B following radiation treatment.

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