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Chapter IV

Systems Biology and Environmental Exposures

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Abstract

Mounting evidence shows that the altered signaling of critical cellular pathways plays a major role in environmental toxicant exposure-induced disease. The current understanding of systems level perturbations caused by exposure to environmental toxicants continues to grow. Here we summarize the literature relating pathway responses to six important toxicants, namely arsenic, benzene, cadmium, chromium, cigarette smoke, and formaldehyde. By integrating the data presented in the current review, we identify the "Environmental Toxicant Signalisome", a network containing pathways that are commonly responsive to varied environmental exposures in mammals. This chapter provides an overview of signaling pathways that are known to be modulated by environmental toxicants of relevance to human exposure and disease.

Keywords: Cell signaling; environmental pollutants; exposure; pathways; systems biology

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1. Introduction

The field of systems biology represents an expanding branch of research focused on understanding complex interactions within biological systems. Systems biology research typically integrates the results obtained using high-throughput technologies with known molecular/protein cellular interactions. The aim is generally to gain insight into signaling events and overall cellular impacts. Such an integration of experimental and computational approaches is important to the discovery and interpretation of complex biological interactions and responses to environmental toxicant exposure.

Systems biology has been a growing topic of investigation since the mid-1990's, alongside the increased information on the sequence of the human genome and the advent of high-throughput technologies (Southern et al., 1999). These high-throughput technologies have allowed the rapid and economical assessment of genome-wide, or 'omics'-based, endpoints. Unlike more traditional molecular biology strategies in which the function(s) of individual genes and gene products are investigated, omics-based strategies provide non-targeted identification of many to all genes or gene products (Ge et al., 2003). This non-targeted strategy allows for unbiased identification of novel mediators of response to a toxicant or association with a disease, which can be overlaid onto molecular networks to identify related signaling pathways at the systems biology level.

One of the most widely applied genome-wide endpoints is gene or messenger RNA (mRNA) expression, which can be evaluated using high-density arrays of nucleic acids (also referred to as DNA microarrays or GeneChip arrays) (Lockhart et al., 2000) or RNA sequencing methods (Ozsolak et al., 2009). These methods measure the relative levels of genes that are expressed or transcribed from genomic DNA through the evaluation of mRNA abundance. The endpoint evaluated using this methodology is often termed a gene expression profile or the 'transcriptome' of a sample of interest (Lockhart et al., 2000). The transcript levels are assessed as the precursors of functional proteins in the cell.

Ideally, systems biology would assess active protein expression in the cell, and indeed protein levels are another endpoint used in systems biology-based studies. Proteomics, or the large-scale study of protein expression levels (Pandey et al., 2000), is extremely valuable to investigate and has clear advantages in comparison to transcriptomics. For example, there are instances when mRNA levels may not correlate directly with protein levels (Greenbaum et al., 2003). This can occur as a result of various post-transcriptional regulatory mechanisms involved in translating mRNA into protein (Greenbaum et al., 2003). Poor mRNA-protein level correlations may also result from a difference in degradation half-lives between mRNAs and proteins (Greenbaum et al., 2003). Still, in situations where correlations between mRNA and protein expression are only weakly positive for individual loci, overall strong correlations have been observed when looking at pathways and complexes of proteins that function together (Washburn et al., 2003).

Because proteins represent the most relevant biological output, it is informative to assess their levels when evaluating cellular interactions at a systems level. There are several methodologies used to perform proteomics-based analyses, including two-dimensional gel electrophoresis, protein chips, and mass spectrometry (Cox et al., 2011; Fenselau 2007; Pandey et al., 2000). The main disadvantage to the proteomics evaluation is that technological limitations currently exist. Specifically, all the proteins expressed in a mammalian cell or

tissue have yet to be identified or measured (Cox et al., 2011). Still, scientists are currently able to measure the levels of thousands of proteins (Cox et al., 2011), which provide important insight into proteomic responses to environmental contaminants.

Some of the earliest systems biology-based research was performed using yeast models. A pioneering study was performed in 1999 by Jelinksy and Samson which evaluated global transcriptional responses of *Saccharomyces cerevisiae* to the alkylating agent, methyl methanesulfonate (Jelinsky et al., 1999). The researchers found that in response to the DNA damaging agent, *S. cerevisiae* not only regulated genes involved in DNA repair, but also genes involved in other cellular processes (Jelinsky et al., 1999). The Samson laboratory expanded their findings by investigating *S. cerevisiae* exposed to several different toxicants, including carcinogenic alkylating agents, oxidizing agents, and ionizing radiation (Jelinsky et al., 2000). Examining the transcriptional responses to these agents revealed both agent-specific transcriptional profiles and transcriptional profiles that were common to multiple exposures (Jelinsky et al., 2000). A general stress response pathway in *S. cerevisiae* was identified in a study performed by Gasch et al. in 2000 (Gasch et al., 2000) which was uncovered through the evaluation of transcriptional responses induced by various environmental stressors (Gasch et al., 2000). These environmental stressors were found to modify the levels of distinct groups of transcripts specific to each stress in addition to a set of transcripts responsive across all stresses, referred to as "environmental stress response" genes (Gasch et al., 2000). The genes that were suppressed in the environmental stress response gene set included those involved in growth-related processes, RNA metabolism, nucleotide biosynthesis, secretion, and those encoding ribosomal proteins. Genes that were induced in the environmental stress response gene set included those involved in carbohydrate metabolism, detoxification of reactive oxygen species, cellular redox reactions, cell wall modification, protein folding and degradation, DNA damage repair, fatty acid metabolism, metabolite transport, autophagy, and intracellular signaling (Gasch et al., 2000).

Systems biology-based approaches have been used for a variety of applications, including cancer prognosis (Sotiriou et al., 2003), drug discovery (Butcher et al., 2004), and exposure risk assessment (Edwards et al., 2008). Investigations involving systems biology have also been performed to increase the understanding of the relationship between exposure to environmental toxicants and disease development. As an example, our laboratory recently used a systems biology approach to identify a key pathway required for the prevention of metal-induced birth defects (Ahir et al., 2013). These studies provide important knowledge on the functioning of biological systems and the exciting opportunity to potentially intervene in the disease process.

1.1. Chapter Aim and Organization

In this review, we summarize the current status (as of January 2013) of research investigating the effects of exposure to selected environmental toxicants/pollutants at the systems biology level. While a comprehensive summary of all systems biology-related research that has investigated the effects of environmental exposures was of interest, a prioritization was made to select contaminants with high relevance to human health and disease and high ranking amongst environmental toxicants. Thus, in order to be included in this review, studies must have implemented large-scale approaches, most commonly through

the measurement of expression levels across more than 100 genes or proteins. In addition, the investigators must have related their findings to effects at the systems-level. For example, studies were required to: (1) comment on functional gene/protein groups that were enriched amongst the exposure-modulated genes or proteins, and/or (2) identify signaling pathways that were likely modulated by the exposure. Studies using humans, mammalian models, and/or mammalian or human cell cultures were included.

Understanding the mechanisms underlying environmental exposure-induced disease is of great importance. The World Health Organization estimates that between 13% and 37% of global disease burden is attributable to environmental factors (i.e. indoor air pollution, outdoor air pollution, and unsafe water, sanitation, and hygiene) (Prüss-Ustün et al., 2008). Understanding the biological factors mediating responses to environmental exposures is therefore of utmost importance. With increased knowledge regarding the biological responses to environmental exposures, accurate exposure limits can be put in place to protect human health. Furthermore, with this knowledge methods can be developed to effectively treat or prevent environmental exposure-induced disease. A thorough understanding of the consequences caused by environmental exposures can ultimately protect public health and alleviate the burden of disease caused by environmental factors.

In this review, we first focus on the systems-level effects of environmental air pollutants (section 2). It is important to understand mechanisms linking exposure to air pollutants to disease, as indoor and outdoor air pollutants significantly contribute to worldwide morbidity and mortality (Bruce et al., 2000; Brunekreef et al., 2002). Here, we focus on air pollutants for which there are systems biology-related findings. Air pollutants were selected that occur as common exposures present throughout indoor and outdoor environments. Specifically, benzene and formaldehyde were selected for review as they are ranked within the Agency for Toxic Substances and Disease Registry (ATSDR) 2011 Substance Priority List (ATSDR 2011), and they are both categorized by the Environmental Protection Agency (EPA) as hazardous air pollutants (EPA 2008). Cigarette smoke (CS) was selected as another air pollutant to review since a large proportion of adults are current smokers. For instance, in 2009, approximately 46.6 million people, or 20.6% of the adults in the United States smoked cigarettes (ALA 2011).

The second focus of this review is on the systems biology responses resulting from exposure to metals or metalloids (section 3). The chemicals prioritized for this section were selected as those with the greatest amount of literature and the highest ranking within the ATSDR 2011 Substance Priority List. Specifically, arsenic, cadmium, and chromium were selected as many systems-level investigations have been performed on these metals/metalloids. Arsenic, cadmium, and chromium are also ranked 1st, 7th, and 17th in the ATSDR 2011 Substance Priority list, respectively, indicating their high potential for toxicity, widespread human exposure, and public concern (ATSDR 2011).

2. Effects of Air Pollutants at the Systems-Level

2.1. Benzene

Benzene is a hazardous air pollutant and a known human carcinogen (IARC 2012a). Within outdoor atmospheres, benzene is released from biogenic sources, including forest fires and oil seeps, and anthropogenic sources, including automobile exhaust, industrial emissions, and evaporations from automobile fueling stations (NTP 2011). Benzene is an important industrial chemical used as a solvent in chemical and pharmaceutical industries and as a starting compound and intermediate during the synthesis of many chemicals (NTP 2011). Because of its prevalence within industrial settings, workers can potentially be exposed to high levels of benzene. These occupational exposures can occur in industries related to petroleum refining, shipping, automobile repair, firefighting, rubber manufacturing, and shoe manufacturing (ATSDR 2007a). Benzene is also present in cigarette smoke, where the amount of benzene within mainstream smoke has been measured to range between 5.9 to 73 μg/cigarette (Brunnemann et al., 1990). Larger amounts of benzene can be present in sidestream smoke, or smoke that is released from the burning end of a cigarette, which has been measured to contain between 345 to 653 μg/cigarette (Brunnemann et al., 1990). Average smokers are estimated to take in 10 times the amount of benzene than nonsmokers on a daily basis (ATSDR 2007a).

Epidemiological studies have shown that benzene exposure is associated with various hematological diseases, including acute myeloid leukemia, myelodysplastic syndromes, non-Hodgkin lymphoma, and childhood leukemia (Smith 2010). In order to gain an understanding of the biological events that link benzene exposure to these serious health effects, studies have been performed evaluating the effects of benzene exposure at the systems biology level.

2.1.1. In Vitro Assessment of Benzene

The effects of benzene and benzene metabolites have been evaluated in human leukocytes *in vitro*. For example, Gillis et al. in 2007 used cytokine/chemokine multiplex assays to find that exposure to the benzene metabolites catechol, hydroquinone, 1,2,4-benzenetriol, and p-benzoquinone stimulated the production of chemokines and cytokines in human peripheral blood mononuclear cells (Gillis et al., 2007). The stimulated cytokines included the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), as well as the Type 2 helper T cell (Th2) cytokines IL-4 and IL-5. A toxicogenomic assessment of responses to 1,2,4-benzenetriol revealed that genes involved in the regulation of protein expression were suppressed, and genes that encode heat shock proteins and cytochrome P450 family members were activated by this metabolite (Gillis et al., 2007). In 2011, Sarma et al. investigated toxicogenomic responses to benzene and the benzene metabolites hydroquinone and 1,4-benzoquinone in human promyelocytic leukemia blood cells (Sarma et al., 2011). The genes that were differentially expressed after exposure to benzene or its metabolites were identified as functionally enriched for apoptosis, cell cycle, tumor protein p53 (p53) signaling, mitogen-activated protein kinase (MAPK) signaling, and T cell receptor (TCR) signaling. The modified gene expression profiles and enriched pathway signaling may provide insight into leukemogenesis (Sarma et al., 2011).

2.1.2. Assessment of Benzene Using Rodent Models

In order to assess the effects of benzene inhalation exposure *in vivo*, benzene exposure has been evaluated using experimental animals. For instance, in 2004, Lee et al. assessed the levels of ~1000 proteins within the plasma of rats exposed to benzene (Lee et al., 2004). Using two-dimensional gel electrophoresis, the investigators identified several proteins at increased levels in benzene-exposed rats, including T cell receptor alpha chain (Lee et al., 2004). In 2010, a study was performed by Hirabayashi and Inoue in which global gene expression analysis of bone marrow cells in mice exposed to benzene revealed that myocyte enhancer factor 2C (*Mef2c*) is commonly decreased at the expression level (Hirabayashi et al., 2010). Benzene-induced expression of integrin beta 2 and the Runx family was also observed. These findings are of biological interest, as the proteins encoded by the benzene-altered genes play important roles in lymphocyte differentiation, hematopoietic progenitor cell proliferation, and the stabilization of hematopoietic niches (Hirabayashi et al., 2010).

2.1.3. Assessment of Benzene in Humans

Two studies have assessed the systems-level effects of benzene inhalation exposure in humans. The first study, performed in 2005 by Forrest et al. assessed global gene expression profiles within peripheral blood mononuclear cells of shoe factory workers (Forrest et al., 2005). A statistical comparison of gene expression profiles between unexposed versus benzene-exposed workers revealed that benzene exposure significantly altered the expression levels of chemokine (C-X-C motif) ligand 16 (*CXCL16*), zinc finger protein 331 (*ZNF331*), jun proto-oncogene (*JUN*), and platelet factor 4 (*PF4*). These genes were then implicated as potential biomarkers of benzene exposure (Forrest et al., 2005). Taking this assessment further, an additional study was performed by the same group in 2009 which assessed global gene expression profiles in peripheral blood mononuclear cells of shoe factory workers (McHale et al., 2009). A similar analytical comparison of exposed versus unexposed workers confirmed the disruption of *CXCL16*, *ZNF331*, *JUN*, and *PF4* induced by benzene inhalation exposure. A gene ontology analysis of all the benzene-associated genes revealed a significant enrichment of genes involved in apoptosis and pathway signaling related to lipid metabolism (McHale et al., 2009).

2.1.4. Summary of Systems-level Findings for Benzene

Comparing the selected systems biology-based findings reveals that certain pathways and cell functions are altered by exposure to benzene and/or benzene metabolites. Specifically, two biological functions were identified as associated with benzene and/or benzene metabolites within two or more studies: apoptosis and cytokine production (Table 1). These functions represent responses that are commonly induced within the blood by benzene regardless of the species or experimental model examined. Apoptosis is a critical function needed to properly regulate cell growth, where impaired apoptosis can lead to cellular transformation and cancer development (Hanahan et al., 2011). Cytokine production occurs in cells that are undergoing stress/inflammation, where released cytokines can perform a variety of functions. For instance, cytokines can activate antiviral activities, stimulate the proliferation and differentiation of blood cells, stimulate major histocompatibility complex antigen expression, stimulate chemotaxis of leukocytes, and activate phagocyte killing of bacteria, fungi, parasites, and tumor cells (Liles et al., 1995).

Across the summarized studies, three signaling pathways were identified as associated with benzene exposure: MAPK signaling, p53 signaling, and TCR signaling (Table 2). Notably, TCR signaling was identified as altered by benzene exposure in two of the six reviewed studies (Lee et al., 2004; Sarma et al., 2011). TCR signaling is an established pathway that signals naïve T cells to activate, generating effector T cells through the onset of rapid proliferation and production of effector cytokines (Huang et al., 2004). It is important to note that the TCR signaling pathway causes changes in gene expression that are dependent upon three key transcription factors: nuclear factor kappa B (NFκB), nuclear factor of activated T cells (NFAT), and activating protein-1 (AP-1), which result in the production of effector cytokines (Huang et al., 2004). Once activated, T cells play important roles in adaptive immune response, but their inappropriate activation can cause autoimmune diseases or cancer (Haugen et al., 2004; Pentcheva-Hoang et al., 2009).

Table 1. Biological functions associated with exposure to benzene and/or benzene metabolites. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Apoptosis	*in vitro*	Sarma 2011
	human	Forrest 2005, McHale 2009
Cytokine production	*in vitro*	Gillis 2007
	human	Forrest 2005, McHale 2009

Table 2. Pathways associated with exposure to benzene and/or benzene metabolites

Enriched Pathway	Model	Reference(s)
MAPK signaling	*in vitro*	Sarma 2011
p53 signaling	*in vitro*	Sarma 2011
TCR signaling	*in vitro*	Sarma 2011
	rodent	Lee 2004

2.2. Cigarette Smoke (CS)

Exposure to cigarette smoke (CS) is estimated to cause 443,000 premature deaths and $96.8 billion in productivity losses per year in the United States alone (CDC 2008). CS is an extremely harmful mixture of gas-phase and particulate toxicants known to induce adverse health effects not only in smokers but also individuals exposed to second hand smoke (Stämpfli et al., 2009). Individuals chronically exposed to CS experience increased risk of developing asthma, chronic obstructive pulmonary disease (COPD), cardiovascular disease, and lung cancer, as well as increased risk of respiratory bacterial/viral infections (Stämpfli et al., 2009; Taylor 2010). Because of the serious health consequences of CS exposure, many studies have been performed to increase the understanding of mechanisms linking CS exposure to disease. A number of these studies have evaluated the impact of CS exposure at the systems biology level.

2.2.1. In Vitro Assessment of CS

The systems-level impact of CS exposure has been extensively evaluated using *in vitro* models. For instance, in 2002, Bosio et al. used spotted arrays containing 513 different cDNA probes to evaluate the transcriptional effects of CS extract exposure in fibroblast cells (Bosio et al., 2002). CS extract was found to increase the expression levels of genes involved in antioxidant response, cell cycle regulation, inflammation, and genes encoding transcription factors. CS extract was also found to increase and decrease the expression levels of genes involved in immune response (Bosio et al., 2002).

In 2005, Han et al. identified members of the cytochrome P450 family (*CYP1A1* and *CYP1B1*) as the genes most up-regulated at the expression level by CS extract in normal bronchial epithelial cells (Han et al., 2005). When this finding was further assessed, estrogen receptor alpha (ERα) was found to regulate CS extract-induced *CYP1B1* expression at the transcriptional level and CYP1A1 expression at the protein level (Han et al., 2005). Another study performed in 2005 by van Leeuwen et al. evaluated the impact of CS condensate on gene expression profiles in human peripheral blood mononuclear cells (van Leeuwen et al. 2005). CS condensate was found to significantly alter the expression levels of many genes involved in immune response and stress response. Interestingly, no genes involved in DNA damage response were affected by CS condensate (van Leeuwen et al., 2005).

In 2006, Nagaraj et al. exposed human oral cells to CS condensate and evaluated their transcriptional responses using high-density microarray RNA expression profiling (Nagaraj et al., 2006). Some of the genes showing the highest increased expression resulting from CS condensate exposure included members of the cytochrome P450 family (*CYP1A1*, *CYP1B1*) and aldo-keto reductase family (*AKR1C1*, *AKR1C3*, *AKR1B10*) (Nagaraj et al., 2006). It was suggested these genes be used in future investigations to determine the severity of CS exposure (Nagaraj et al., 2006).

An investigation performed in 2007 by Maunders et al. employed a three-dimensional model of the human tracheobronchial epithelium and exposed cells to whole mainstream CS at a direct air-liquid interface (Maunders et al., 2007). Genome-wide expression analysis revealed that genes differentially expressed upon exposure to CS were enriched for many cellular processes, including DNA damage and repair, oxidant/antioxidant balance, and xenobiotic metabolism. After 1 hour of CS exposure, there was a down-regulation of genes in the transforming growth factor-beta (TGFβ) pathway. After 6 hours of CS exposure, there was an up-regulation of genes involved in the MAPK pathway. Also of note, two of the genes showing the highest increase in expression resulting from CS exposure were *CYP1A1* and *CYP1B1* (Maunders et al., 2007). Results from this study provided a greater understanding of smoke toxicity within the mucociliary epithelium.

Also in 2007, Lu et al. evaluated genomic responses to whole smoke from cigarettes with varying tar content in mouse fibroblast cells (Lu et al., 2007). The high tar cigarette exposure decreased the expression levels of genes involved in DNA replication and cell proliferation. The decreased expression of genes involved in cell proliferation was of interest, as the investigators noted that decreased cell proliferation is a proposed mechanism of emphysema development in smokers. All of the tested cigarettes increased the expression levels of genes related to glutathione biosynthesis/consumption and inflammatory response, which are known biological responses to CS. The genes with the highest increased expression levels in smoke-exposed cells were *Cyp1a1*, glutathione S-transferase alpha 2 (*Gsta2*), heme oxygenase (decycling) 1 (*Hmox1*), and protein C receptor, endothelial (*Procr*). These genes were

implemented as new biomarkers for assessing and monitoring the biological effects of CS (Lu et al., 2007).

In 2008, Gümüş et al. evaluated the transcriptomic changes resulting from CS extract in MSK-Leuk1 cells, a cell model of oral leukoplakia (Gümüş et al., 2008). Pathways related to apoptosis, cell proliferation, inflammation, and tissue injury were modified by CS exposure. Network analysis revealed that CS extract induced several epidermal growth factor receptor (EGFR) ligands along with several aryl hydrocarbon receptor (AHR)-dependent genes, including those encoding xenobiotic metabolizing enzymes CYP1A1 and CYP1B1. This study's results offer insight into the mechanisms of CS-induced cancer and suggest that inhibitors of EGFR or AhR signaling may prevent or delay the development of CS-related tumors (Gümüş et al., 2008).

In 2010, López-Boado et al. found that rat tracheal explants exposed to CS for 15 minutes exhibited altered expression profiles of genes involved in antioxidant protection, epithelial defense and remodeling, and inflammatory response (López-Boado et al., 2010). Even after the brief exposure to CS, rapid changes in the expression levels of many genes occurred (López-Boado et al., 2010). Lastly, a study performed in 2011 by Sexton et al. used isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labeling techniques to assess the protein expression profiles of airway epithelial cells exposed to CS at a direct air-liquid interface. The researchers identified 466 potential protein biomarkers of CS exposure, including proteins related to lung injury and mechanistic pathways of disease (Sexton et al., 2011).

2.2.2. Assessment of CS Using Rodent Models

Effects of CS exposure have also been evaluated at the mechanistic level using several *in vivo* models. For example, in 2004 Rangasamy et al. identified the nuclear factor, erythroid-derived 2, like 2 (NRF2) as a critical transcription factor involved in the mediation of phenotypic and genomic responses to CS in mice (Rangasamy et al., 2004). *Nrf2*-knockout mice were found to exhibit earlier-onset and more extensive CS-induced emphysema in comparison to wild-type mice with functional *Nrf2*. Comparing the gene expression profiles of *Nfr2*-knockout versus wild-type mice exposed to CS revealed 50 NRF2-dependent antioxidant and cytoprotective genes in the lung that were postulated to counteract CS-induced oxidative stress and inflammation. The results suggest that responsiveness of the Nrf2 pathway may play a role in susceptibility to CS-induced emphysema (Rangasamy et al. 2004).

A similar study was also performed in 2004, where Izzotti et al. compared wild-type mice versus mice with *P53* mutation exposed to CS (Izzotti et al., 2004). Both wild-type and mutant mice responded to CS exposure in a similar manner, where genes involved in metabolism of xenobiotics, multidrug resistance, DNA repair, and protein repair were altered at the expression level. Also common to wild-type and *P53* mutant mice was the altered expression of oncogenes and tumor suppressor genes resulting from CS exposure. The *P53* mutant mice additionally had a lack of induction of proapoptotic genes and an overexpression of genes involved in cell proliferation, signal transduction, angiogenesis, inflammation, and immune response after exposure to CS. The results suggest that the additional responses to CS exposure in the *P53* mutant mice may explain potential differences in susceptibility to CS-induced lung cancer (Izzotti et al., 2004).

A study performed by Gebel et al. in 2004 evaluated gene expression profiles in the respiratory nasal epithelium and lung tissue of rats exposed to CS (Gebel et al., 2004). The most drastic CS-induced differential gene expression occurred in the nasal epithelium of rats after one exposure, where there was increased expression of genes encoding oxidative stress-responsive and Phase II drug-metabolizing enzymes. The investigators noted that these prominently up-regulated genes are all known, at least in part, to be transcriptionally regulated by NRF2. This portion of genomic response appeared to be transient, as the CS-induced increased expression of these oxidative stress and Phase II-responsive genes markedly reduced after three weeks of CS exposure. In contrast, CS exposure caused a sustained increase in expression of genes encoding the Phase I drug-metabolizing enzymes cytochrome P450 CYP1A1 and aldehyde dehydrogenase-3 in both the rat nasal epithelium and lung (Gebel et al., 2004).

The first study to investigate the effects of CS at the proteomic level was performed in 2005 by Izzotti et al. (Izzotti et al., 2005). The researchers used an antibody microarray to measure the levels of 518 proteins within the rat lung, where 56 proteins were identified as significantly increased by CS exposure. The investigators noted that the proteins stimulated by CS were related to apoptosis, cell replication, immune response, phagocytosis, protein removal, and stress response. Using a cDNA microarray, a significant correlation was identified between gene expression intensity and protein levels in both unexposed and exposed rats. The observed proteomic and genomic alterations reflect mechanisms that may contribute to the pathogenesis of smoke-related diseases (Izzotti et al., 2005).

In 2006, Meng et al. found that mice exposed to CS exhibited differential expression of genes within the lung (Meng et al., 2006). These genes were related to decreased heat-shock response and chaperone activity, increased immune and inflammatory response, and increased mitosis. The results suggest that the CS-associated genes and functions may be related to COPD pathogenesis (Meng et al., 2006).

Prenatal exposure to CS was evaluated at the transcriptomic level in 2007 by Rouse et al. (Rouse et al., 2007). An association was identified between *in utero* CS exposure and altered gene expression profiles within the lungs of adult mice. Genome-wide microarray analysis revealed that *in utero* CS exposure disrupted the expression levels of genes related to apoptosis, cell metabolism, cell proliferation, and immune response within the lungs of developed mice. Because the investigators were interested in whether *in utero* exposure to CS influenced the expression levels of asthma-related genes, an alternative gene-specific technique, quantitative real-time polymerase chain reaction (qRT-PCR), was performed and four asthma-related genes were identified at increased expression levels in exposed mice: arginase (*Arg1), chemokine (C-C motif) ligand 24* (*Ccl24)*, mast cell protease 1 (*Mcpt1*), and solute carrier family 7, member 2 (*Slc7a2).* The CS-induced differential gene expression was further implicated in differential respiratory and immune responses to nontobacco allergens (Rouse et al., 2007).

In 2008, Taylor et al. employed information-theoretic algorithms to predict direct transcriptional regulatory interactions mediated by NRF2 in the mouse lung under conditions of oxidative stress (Taylor et al., 2008). These predictions were tested at the gene expression level through microarray analysis of lung tissue from *Nrf2*(+/+) and *Nrf2*(-/-) mice exposed to CS. New potential regulatory loops were identified, and involved NRF2 and its downstream target, NAD(P)H dehydrogenase quinone 1 (NQO1), among others. This research exemplified how to integrate network interference algorithms with high-throughput gene

expression data to identify transcriptional regulatory loops and other signaling relationships possibly involved in disease development (Taylor et al., 2008).

A study carried out in 2009 by Cavarra et al. found that different clusters of genes respond to CS exposure when comparing between various strains of mice (Cavarra et al., 2009). Two strains of smoke-sensitive mice, or mice that develop severe lung changes and/or emphysema after chronic exposure to CS, were assessed alongside a smoke-resistant strain of mice that does not develop emphysema after chronic CS exposure. After four weeks of CS exposure, the lungs of smoke-sensitive mice were found to exhibit altered expression levels of genes related to acute phase response, cell adhesion, and pro-emphysematous. These clusters of genes were not altered by CS within the lungs of smoke-resistant mice. The researchers concluded that the observed differences in response to CS are dependent upon genetic background and likely involve different molecular signaling pathways (Cavarra et al., 2009).

Gebel et al. provided further evidence for the protective role of the Nrf2 pathway in CS-induced oxidative stress and tissue damage in 2010 (Gebel et al., 2010). This study compared genomic responses to chronic CS exposure within the lungs of *Nrf2*(-/-) mice versus wild-type mice. In *Nrf2*(-/-) mice, CS exposure was found to impair the expression of antioxidant and phase 2-related genes. A transcriptomics-based comparison of *Nrf2*(-/-) versus wild-type mice revealed an attenuated cell cycle/mitotic response and increased stress response in CS-exposed *Nrf2*(-/-) mice. These transcriptional responses coincided with clear alveolar destruction and impaired lung function in CS-exposed *Nrf2*(-/-) mice. Both types of mice showed similar inflammation-related transcriptional responses and phenotypic bronchoalveolar inflammation. These findings suggested that CS-induced emphysema is more likely influenced by cell loss and tissue regeneration than by increased inflammation (Gebel et al., 2010).

In 2011, Carter et al. used a proteomics analysis to evaluate the expression levels and phosphorylation of 650 proteins using antibody microarray technology (Carter et al., 2011). Proteins were assessed in the lungs of rats exposed to mainstream whole CS. Proteins known to regulate apoptosis, cell structure, inflammation, and stress responses were identified as altered by CS exposure. These changes were implicated as early indicators of lung damage (Carter et al., 2011).

2.2.3. Assessment of CS in Humans

The effect of CS exposure on biological signaling pathways has been assessed in humans. In 2004, Spira et al. performed the first genome-wide transcriptional study in humans involving CS, where gene expression profiles within the bronchial airway epithelium were compared between current smokers and healthy subjects that never smoked. Smoking was found to impact gene expression profiles with a total of 97 genes changing as a result of CS exposure. Genes identified as increased in expression in smokers compared to nonsmokers were involved in the regulation of oxidative stress (e.g. *ALDH3A1*), electron transport, glutathione metabolism, xenobiotic metabolism, and secretion. Genes with decreased expression levels in smokers were related to inflammation. Also of note, several oncogenes were increased in expression and several tumor suppressor genes were decreased in expression in smokers. These findings suggest that large airway epithelial cells likely serve antioxidant, metabolizing, and host-defense functions (Spira et al., 2004).

In 2006, Plymoth et al. compared protein expression profiles within bronchoalveolar lavage (BAL) fluid samples from lifelong smokers versus never-smokers using a shotgun

sequencing approach (Plymoth et al., 2006). Analysis of BAL fluid was performed, as proteins within BAL fluid arise from diverse origins and may act as novel pathological mediators. Using linear ion trap quadropole (LTQ) mass spectrometery, 481 proteins with varying abundances were identified within BAL samples. Proteins identified at modified levels in lifelong smokers were assessed according to gene ontology categorization, where the CS-associated proteins showed a broad distribution of protein functional classes associated with various biological processes, including cell communication, localization, metabolism, organ development, and response to stimulus. These proteins may enable more accurate characterization of various lung diseases at the molecular level (Plymoth et al., 2006).

Woenckhaus et al. identified a gene expression signature within the bronchial epithelium that may reflect early CS-induced responses related to cancer development in smokers in 2006 (Woenckhaus et al., 2006). A transcriptomic comparison between non-smokers, smokers, and patients with non-small-cell lung cancers revealed a signature comprising of 23 differentially expressed genes which may represent early CS-induced and cancer-related changes. This signature comprised genes involved in cell differentiation, matrix degradation, redox stress, and xenobiotic metabolism (Woenckhaus et al., 2006).

A study carried out in 2007 by Harvey et al. compared gene expression profiles of phenotypically normal smokers versus nonsmokers within the small airway epithelium, the region of the airway from which COPD commonly originates (Harvey et al., 2007). Although the smokers were phenotypically normal, transcriptional analysis revealed CS-associated alterations in the expression levels of genes involved in COPD pathogenesis, including genes related to apoptosis, cytokine release / innate immunity, mucin, response to oxidants, and response to xenobiotics. These transcriptional changes in the small airway epithelium are candidates for future therapeutic strategies to prevent COPD onset (Harvey et al., 2007).

Also in 2007, van Leeuwen et al. evaluated the effects of CS exposure on gene expression profiles within the blood of nine smoking-discordant monozygotic twin pairs (van Leeuwen et al., 2007). Using these subjects enabled the accurate assessment of CS-induced effects while reducing influences of varying genetic backgrounds. Gene expression profiles were assessed using cDNA microarrays containing 600 genes from toxicologically relevant categories, including apoptosis, cell cycle, proliferation, biotransformation and metabolism, and inflammation. Genes identified as significantly modified at the expression level by CS exposure were functionally related to anti-apoptosis, carcinogen metabolism, and oxidative stress response (van Leeuwen et al., 2007).

In order to expand the 2004 findings by Spira et al. (Spira et al., 2004), the same group assessed genome-wide transcriptional relationships between the nasal, buccal, and bronchial epithelium of current smokers and never smokers in 2008 (Sridhar et al., 2008). CS-associated increased expression of genes involved in oxidoreductase and electron transport was found in all three of the evaluated airway epithelial regions. Also in common was the CS-associated decreased expression of genes involved in cell motility, cell migration, development, and localization of cellular complexes. These findings suggest that there is a relationship between genomic responses to CS throughout the airway epithelium (Sridhar et al., 2008).

In 2010, Boyle et al. compared genome-wide transcriptional profiles of the oral mucosa of current smokers versus never smokers (Boyle et al., 2010). Smoking was found to alter the expression levels of 41 genes. Of the CS-associated genes, 32 were up-regulated in expression, and were related to cell adhesion, eicosanoid synthesis, nicotine signaling,

oxidant stress, and xenobiotic metabolism. A biological pathway analysis revealed the likely involvement of AhR signaling and Nrf2 signaling in CS-induced responses. Also of note, smoking caused a greater induction of aldo-keto reductases in women in comparison to men. These findings offer insight into the carcinogenic effects of CS and provide knowledge that may be useful towards the development of cancer prevention strategies (Boyle et al., 2010).

An exceptionally large epidemiological study on smoking was performed by Charlesworth et al. in 2010 (Charlesworth et al., 2010). This study assessed genome-wide transcriptional profiles within leukocyte samples collected from 1,240 individuals, 297 of which were current smokers. A group of 323 genes was identified with expression levels that significantly correlate with smoking behavior. These CS-associated genes were enriched for many functional categories, including cancer, cell death, immune response, natural killer cell signaling, and xenobiotic metabolism. This study provided a comprehensive assessment of CS-induced effects on the human transcriptome through a large cross-sectional data set (Charlesworth et al., 2010).

Another comparison between nasal and bronchial epithelial cell responses to CS was performed in 2010 by Zhang et al. (Zhang et al., 2010). Supporting findings from the Sridhar et al. 2008 study (Sridhar et al., 2008), this study identified 119 genes that showed similar differential expression in smokers versus nonsmokers within both the bronchial and nasal epithelium. These commonly altered genes were highlighted for their roles in detoxification, oxidative stress, and wound healing. These data suggest that CS exposure causes largely similar gene expression changes in both the nasal and bronchial epithelium, suggesting that CS effects may be measured in tissues throughout the airway (Zhang et al., 2010).

The effects of chronic CS exposure on the human plasma proteome were recently investigated by Bortner et al. in 2011 (Bortner et al., 2011). This study employed the proteomic approach iTRAQ to identify plasma proteins that were differentially expressed in smokers versus nonsmokers. Of the 113 proteins detected within the plasma, 16 were identified as associated with CS. Several of the CS-associated proteins identified within the plasma play roles in immunity and inflammatory response. These proteins may serve as candidate biomarkers of CS exposure for future epidemiological studies (Bortner et al., 2011).

Beane et al. recently used RNA sequencing-based approaches to further establish transcriptomic responses to CS within the bronchial airway epithelium (Beane et al., 2011). With this approach, the relative expression levels of 20,573 genes were compared between never smokers and current smokers. Pathways relevant to the metabolism of xenobiotics by cytochrome P450, oxidoreductase activity, and retinol metabolism were enriched among the 156 genes modified in smokers. The investigators noted that measuring both coding and non-coding transcripts through RNA sequencing aids in the understanding of responses to CS and in the identification of additional biomarkers of exposure (Beane et al., 2011).

2.2.4. Summary of Systems-level Findings for CS

The effects of CS exposure on cellular signaling and function have been assessed by numerous research groups using cell culture, rodent, and human models. Many of the CS-related findings were consistently reported throughout many studies. These include several biological functions that were identified as associated with genomic and proteomic responses to CS in multiple investigations (Table 3). Many pathways have also been identified as altered by CS exposure (Table 4).

Table 3. Biological functions associated with exposure to CS. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Antioxidant response	*in vitro*	Bosio 2002, Maunders 2007, López-Boado 2010
	rodent	Gebel 2010
Oxidative stress response	*in vitro*	Maunders 2007
	rodent	Gebel 2004
	human	Harvey 2007, van Leeuwen 2007, Boyle 2010, Zhang 2010
Oxidoreductase activity	human	Sridhar 2008, Beane 2011
Apoptosis	*in vitro*	Gümüş 2008
	rodent	Izzotti 2005, Rouse 2007, Carter 2011
	human	Harvey 2007, van Leeuwen 2007
Cell adhesion	rodent	Cavarra 2009
	human	Boyle 2010
Cell proliferation	*in vitro*	Lu 2007, Gümüş 2008
	rodent	Rouse 2007
DNA damage/repair	*in vitro*	Maunders 2007
	rodent	Izzotti 2004
Electron transport	human	Spira 2004, Sridhar 2008
Glutathione biosynthesis/consumption	*in vitro*	Lu 2007
	human	Spira 2004
Immune response	*in vitro*	Bosio 2002, van Leeuwen 2005
	rodent	Izzotti 2005, Meng 2006, Rouse 2007
	human	Harvey 2007, Charlesworth 2010, Bortner 2011
Inflammatory response	*in vitro*	Bosio 2002, Lu 2007, Gümüş 2008, López-Boado 2010
	rodent	Meng 2006, Gebel 2010, Carter 2011
	human	Spira 2004, Bortner 2011
Stress response	*in vitro*	van Leeuwen 2005
	rodent	Izzotti 2005, Carter 2011
Tissue injury	*in vitro*	Gümüş 2008, López-Boado 2010, Sexton 2011
Xenobiotic metabolism	*in vitro*	Maunders 2007
	rodent	Izzotti 2004
	human	Spira 2004, Woenckhaus 2006, Boyle 2010, Charlesworth 2010, Beane 2011

One of the major themes of the CS findings was that exposure causes disruptions in cellular signaling related to xenobiotic metabolism. As cytochrome P450 members regulate xenobiotic metabolism, it is not surprising that CS exposure has been shown to modify the expression levels of cytochrome P450 members (e.g. *CYP1A1*, *CYP1B1*) throughout various model systems (Gebel et al., 2010; Gümüş et al. 2008; Han et al., 2005; Lu et al., 2007; Maunders et al., 2007; Nagaraj et al., 2006).

Table 4. Pathways associated with exposure to CS

Enriched Pathway	Model	Reference(s)
AhR signaling	*in vitro*	Gümüş 2008
	human	Boyle 2010
EGFR signaling	*in vitro*	Gümüş 2008
MAPK signaling	*in vitro*	Maunders 2007
Natural killer cell signaling	human	Charlesworth 2010
Nicotine signaling	human	Boyle 2010
Nrf2 signaling	rodent	Rangasamy 2004, Gebel 2004, Taylor 2008, Gebel 2010
	human	Boyle 2010
p53 signaling	rodent	Izzotti 2004
TGF-β signaling	*in vitro*	Maunders 2007

Originally recognized for its role in the hepatic drug detoxification system, the cytochrome P450 superfamily is now understood to play diverse roles in many enzymatic reactions throughout the body (Nebert et al., 2002). For example, cytochrome P450 members are involved in the metabolism of endogenous substrates as well as exogenous compounds, including drugs, environmental pollutants, and plant products (Nebert et al., 2002). The metabolism of exogenous agents by cytochrome P450 enzymes often results in successful detoxification of the agent. However, in some instances, the action of P450 members can produce toxic metabolites that contribute to increased risks of birth defects, cancer, and other adverse effects (Nebert et al.,2002). Such is the case during the metabolism of CS, where P450 enzymes bioactivate polycyclic aromatic hydrocarbons present in CS causing them to become very toxic substances for the lungs (Ben-Zaken Cohen et al., 2007). For this reason, pathways related to metabolism and cytochrome P450 have been implicated as targets for lung cancer and COPD prevention/treatment (Ben-Zaken Cohen et al., 2007).

A second theme throughout the detailed literature was the involvement of oxidative stress in the systems-level responses to CS exposure. Specifically, biological functions related to antioxidant response, oxidative stress, and oxidoreductase activity were highly enriched for amongst 11 of the 30 CS studies reviewed here. As aldo-keto reductases regulate oxidative stress, it is not surprising that CS exposure has been associated with the altered expression levels of aldo-keto reductase family members throughout multiple model systems (Boyle et al., 2010; Nagaraj et al., 2006). A signaling pathway that was shown to heavily influence cellular response to CS exposure was the Nrf2 pathway. Five *in vivo* studies provided clear evidence that Nrf2 signaling plays a role in the protection against CS-induced adverse effects, including oxidative stress, tissue damage and/or emphysema (Boyle et al., 2010; Gebel et al., 2004; Gebel et al., 2010; Rangasamy et al., 2004; Taylor et al., 2008). Nrf2 signaling is known to coordinate the activation of antioxidant enzymes as well as the expression of antioxidant response element-related genes (Jaiswal 2004), further demonstrating the link between CS exposure and oxidative stress-related signaling.

2.4. Formaldehyde

Formaldehyde is a ubiquitous air pollutant present in both indoor and outdoor atmospheres. In indoor environments, sources of formaldehyde include plywood, furniture, particle-board, certain insulation materials, carpets, paints and varnishes, textiles, tobacco smoke, and the use of formaldehyde as a disinfectant (IARC 2012b; NTP 2011). In outdoor environments, formaldehyde is produced as both a primary and secondary air pollutant via atmospheric photochemistry (IARC 2012b; NTP 2011). Some of the highest formaldehyde exposures occur in occupational settings, such as industries involving resin, plastics, wood, paper, insulation, textile, chemical productions, disinfectants, and embalming products (IARC 2012b; NTP 2011). Formaldehyde is also formed *in vivo* through the metabolism and processing of drugs, dietary agents, and amino acids (O'Brien et al., 2005).

Epidemiological studies have found that formaldehyde is associated with increased risk of childhood asthma, acute respiratory tract illness, sinonasal cancer, nasopharyngeal cancer, and possibly leukemia (IARC 2012b; NTP 2011). In toxicological studies, formaldehyde has been shown to cause nasal squamous cell carcinomas in rats and to a lesser extent, in mice (NTP 2011). Formaldehyde is currently classified by the International Agency for Research on Cancer as a known human carcinogen (IARC 2006). Because of the presence of both endogenous and environmental formaldehyde exposure, coupled with its deleterious health effects, understanding the exposure response and biological basis of formaldehyde-induced health effects is of utmost importance.

2.4.1. In Vitro Assessment of Formaldehyde

In 2010, Neuss et al.,exposed primary human nasal epithelial cells to formaldehyde and assessed alterations in both gene expression profiles and DNA-protein crosslink levels (Neuss et al.,2010). High levels of formaldehyde exposure (100 and 200 μM) were found to significantly induce DNA-protein crosslinks and alter gene expression signatures. To the investigators' surprise, a pathway analysis of the formaldehyde-responsive genes showed that the primary pathways involved in formaldehyde detoxification and DNA-protein crosslink repair were not enriched. Other biological processes were associated with the formaldehyde-responsive genes, including apoptosis, cell death, nucleosome assembly, response to stress, regulation of metabolism, transcription, and translation (Neuss et al., 2010). These data highlight the role that systems biology can play in identifying key molecular targets that would otherwise be unlikely candidates through *a priori* selection.

2.4.2. Assessing Formaldehyde Using Rodent Models

The systems-level effects of formaldehyde inhalation exposure have been evaluated within the rat airway epithelium at the gene expression level. The first study to evaluate the toxicogenomic changes induced by formaldehyde exposure *in vivo* was performed by Hester *et al.* in 2003 (Hester et al., 2003). The researchers exposed rats using a nasal instillation of liquid formaldehyde. A global gene expression analysis of the nasal epithelium revealed that formaldehyde-induced changes in gene expression were related to several functional gene categories, including apoptosis, cell cycle, DNA repair, and xenobiotic metabolism (Hester et al., 2003). A 2007 study performed by Sul *et al.* exposed rats to formaldehyde for two weeks using an inhalation chamber (Sul et al., 2007). This study assessed gene expression profiles within the rat lung tissue, and identified several gene groups as influenced by formaldehyde

exposure. The genes with modified expression levels resulting from formaldehyde exposure were highlighted for their roles in apoptosis, coagulation, immunity, metabolism, oncogenesis, signal transduction, and transportation (Sul et al., 2007).

A study carried out in 2007 by Thomas *et al.,* applied genomic information to risk assessment practices (Thomas et al., 2007). As a case study, investigators assessed gene expression changes resulting from various levels of acute formaldehyde exposure in the rat nasal epithelium. Gene ontology enrichment analyses revealed that different groups of formaldehyde-responsive genes can result from different exposure levels. More specifically, biological processes enriched amongst the genes associated with lower formaldehyde exposure levels included microtubule polymerization, xenobiotic stimulus, and regulation of c-jun N-terminal kinase (JNK) cascade. Biological processes enriched amongst the genes associated with higher formaldehyde exposure levels included response to DNA damage, cell proliferation, and inflammatory response. This investigation exemplified an important advance in the application of genomic information to risk assessment though a comprehensive survey of exposure-induced genomic changes (Thomas et al., 2007).

The same research group expanded these findings in 2008 and 2010 with two genomic studies (Andersen et al., 2008; Andersen et al., 2010). Both of these studies evaluated potential time-dependencies and concentration-dependencies in genomic responses to formaldehyde in the rat nasal epithelium. The 2008 study identified gene ontology enrichment for hundreds of categories across varying exposure conditions. Of note, the highest formaldehyde exposure of 15 ppm was evaluated via inhalation exposure and nasal instillation, where both exposure routes induced the expression of genes involved in response to wounding, control and induction of apoptosis, inflammation pathways, and receptor tyrosine kinase signaling (Andersen et al., 2008). Other gene ontology categories were enriched after 15 ppm formaldehyde inhalation exposure, including cell defense, response to stress, transcription, transmembrane signaling, and NFκB cascades (Andersen et al., 2008). The 2010 study performed by Andersen *et al.,* evaluated longer exposure durations and identified a group of genes responsive to 2 ppm formaldehyde exposure at all durations (Andersen et al., 2010). These genes, labeled "sensitive response genes", were associated with cellular stress, thiol transport/reduction, inflammation, and cell proliferation. At formaldehyde levels of 6 ppm or greater, investigators identified an enrichment of formaldehyde-responsive genes involved in apoptosis, cell cycle, and DNA repair (Andersen et al., 2010). ERBB, EGFR, Wnt, TGF-β, Hedgehog, and Notch-related signaling were also enriched for amongst genomic responses to the higher formaldehyde exposure levels (Andersen et al., 2010). Findings from these *in vivo* studies are critical, as the identified changes in mRNA abundance resulting from formaldehyde exposure provide knowledge useful to define mechanisms underlying formaldehyde-induced cancer.

2.4.3. Summary of Systems-level Findings for Formaldehyde

The effects of formaldehyde exposure on cellular signaling and function have been assessed by many research groups using cell culture and rodent models. Multiple studies found overlapping functions that are modified by formaldehyde exposure. Specifically, two or more studies identified apoptosis, cell cycle, cell proliferation, DNA repair, inflammatory response, metabolism, response to stress, and transcription as biological processes impacted upon exposure to formaldehyde (Table 5). Many of these functional responses identified at the genomic level have also been observed at the phenotypic level. As recently reviewed by

Swenberg and colleagues, inhalation exposure to formaldehyde is known to cause increased cell proliferation in regions that directly contact exogenous formaldehyde (i.e. the nasal epithelium) in various experimental animals (Swenberg et al., 2013). Inhaled formaldehyde is also known to be genotoxic, causing DNA-protein crosslinks, DNA double strand breaks, and various types of DNA adducts (NTP 2011; Swenberg et al., 2013). The consistency between 'omics-based functional enrichment findings and phenotypic observations supports the use of gene or protein expression levels as biomarkers of effect.

Table 5. Biological functions associated with exposure to formaldehyde. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Apoptosis	*in vitro*	Neuss 2010
	rodent	Hester 2003, Sul 2007, Andersen 2008, Andersen 2010
Cell cycle	rodent	Hester 2003, Andersen 2010
Cell proliferation	rodent	Thomas 2007, Andersen 2010
DNA repair	rodent	Hester 2003, Andersen 2010
Inflammatory response	rodent	Thomas 2007, Andersen 2008, Andersen 2010
Metabolism	rodent	Hester 2003, Sul 2007
Stress response	*in vitro*	Neuss 2010
	rodent	Andersen 2008
Transcription	*in vitro*	Neuss 2010
	rodent	Andersen 2008

Table 6. Pathways associated with exposure to formaldehyde

Enriched Pathway	Model	Reference(s)
EGFR signaling	rodent	Andersen 2010
ERBB signaling	rodent	Andersen 2010
Hedgehog signaling	rodent	Andersen 2010
JNK signaling	rodent	Thomas 2007
NFkB signaling	rodent	Andersen 2008
Notch signaling	rodent	Andersen 2010
Receptor tyrosine kinase signaling	rodent	Andersen 2008
TGF-β signaling	rodent	Andersen 2010
Wnt signaling	rodent	Andersen 2010

Several pathways were also identified as modified by formaldehyde exposure using rodent models (Table 6). Pathways responsive to formaldehyde in this review include Hedgehog, Notch, and Wnt signaling. These pathways are major players in the regulation of cell development, cell differentiation, and cell fate determination (Takebe et al., 2011). In cancer cells, Hedgehog, Notch, and Wnt signaling are sometimes modified, contributing to uncontrolled cell growth/differentiation and self-renewal properties (Takebe et al., 2011). For this reason, Hedgehog, Notch, and Wnt signaling are, in some cases, being targeted for cancer treatment (Takebe et al., 2011). Suppression of the Notch signaling pathway, in particular, has

been implicated in nasopharyngeal cancer (Sriuranpong et al., 2004), a type of cancer that is biologically relevant to formaldehyde exposure (Swenberg et al., 2013). Formaldehyde-induced alterations of these systems-level responses may, therefore, represent important mechanistic links between formaldehyde exposure and cancer.

3. Effects of Metals at the Systems-Level

3.1. Arsenic

Arsenic is currently ranked as the #1 priority contaminant of the ATSDR (ATSDR 2011). It is poisoning the water of more than 100 million people around the globe and represents a critical health issue and cause of global disease (Uddin et al., 2011). Inorganic arsenic (iAs) is a known human carcinogen (IARC 2004) that is a naturally occurring semi-metallic element widely distributed throughout the Earth's crust (ATSDR 2007b). It is present in many minerals and can be naturally released into the environment (ATSDR 2007b). Anthropogenic activities, including metal mining and smelting, pesticide application, coal combustion, wood combustion, and waste incineration, also contribute to arsenic accumulation in the environment (ATSDR 2007b).

The general population is exposed to arsenic and arsenic compounds primarily through dietary consumption. For example, high levels of arsenic have been detected in mushrooms, poultry, rice, and seafood (NTP 2011). Some populations are exposed to arsenic through consumption of drinking water that is contaminated with arsenical pesticides or naturally occurring mineral deposits (NTP 2011).

iAs levels above the World Health Organization's recommended limit have been detected in drinking water sources in several areas throughout the world, including Bangladesh, Mexico, India, Vietnam, and in the United States (ATSDR 2007b), including recently reported findings in the state of North Carolina (Sanders et al., 2012). Occupational exposure to iAs can also occur for workers involved in metal smelting, pesticide manufacturing or application, wood preservation, semiconductor manufacturing, or glass production, primarily from dusts or aerosols in the air (ATSDR 2007b).

Cancer tissue sites that have been linked to arsenic exposure include the digestive tract, kidney, liver, lung, skin, urinary bladder, and lymphatic and hematopoietic systems (NTP 2011). Arsenic is not only a human carcinogen, but it is also used in some cases to treat cancer, such as promyelocytic leukemia (NTP 2011). iAs exposure has also been linked to a variety of other health effects, including neurobehavioral effects, adverse effects on memory and intellectual function, heart disease, liver hypertrophy, respiratory system disease, type-two diabetes, and several reproductive effects, including pregnancy complications and toxic effects on developing fetuses (Kapaj et al., 2006). Because of the serious health effects resulting from iAs, coupled with arsenic's potential as a chemotherapeutic, the effects of arsenic exposure/treatment have been studied extensively at the systems biology level.

3.1.1. In Vitro Assessment of Inorganic Arsenic

Disruptions at the systems-level resulting from arsenic exposure have been evaluated in many *in vitro* studies. For example, in 2000, Simeonova et al., treated human bladder

epithelial cells with arsenite, which was found to cause increased cell proliferation alongside increased DNA binding activity of the AP-1 transcription factor (Simeonova et al., 2000). These findings were confirmed *in vivo*, where mice were exposed to 0.01% sodium arsenite in drinking water, and increased AP-1 activity and hyperplasia were found in the bladder urothelium of exposed mice. A transcriptional analysis of the human bladder epithelial cells revealed that arsenite exposure increased the expression levels of genes associated with cell arrest, including *GADD45* (growth arrest and DNA-damage-inducible, alpha) and *GADD153* (DNA-damage-inducible transcript 3), and cell growth, including *EGR-1* (early growth response 1), *FOS* (FBJ murine osteosarcoma viral oncogene homolog), and *JUN*. The results suggest that arsenic's proliferation-enhancing effect, as observed at the transcriptional and phenotypic level, likely contributes to its carcinogenicity (Simeonova et al., 2000).

The Waalkes laboratory in 2001 performed the first study to associate disruptions in gene expression profiles with iAs-induced malignant transformation (Chen et al., 2001). In this study, normal rat liver cells were chronically treated with low levels of arsenite, and after 18 weeks, the cells transformed into malignant cells. Out of the 588 genes assessed, ~80 were aberrantly expressed in arsenic-transformed cells. Arsenic-transformed cells were found to exhibit alterations for genes involved in apoptosis, cell cycle regulation, cytokine production, growth-factor and hormone-receptor production, signal transduction, and stress response. In addition, the expression levels of several oncogenes were increased in arsenic-transformed cells. This analysis revealed altered gene expression patterns associated with arsenic-induced malignant transformation, acting as an important basis for future investigations (Chen et al., 2001).

In 2002, Bae et al*.,* evaluated human keratinocytes transformed by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and then exposed to arsenic (Bae et al., 2002). Transcriptional analysis was performed after arsenic exposure, where multiple genes encoding for DNA repair proteins were found to be overexpressed in human keratinocytes exposed to arsenic. This type of mechanistic study provides knowledge on the relationship between altered gene expression and various states of carcinogenesis (Bae et al., 2002).

Also in 2002, Yih et al*.,* exposed human fibroblast cells to 5 μM sodium arsenite for up to 24 hours (Yih et al., 2002). Assessing the transcript levels of 568 genes through various time points, 133 genes were identified as showing significant, temporally-related expression changes in the presence of arsenite compared to unexposed time-matched controls. Of the 133 arsenite-associated genes, the expression levels of 94 genes were increased by arsenite while 39 were decreased. Gene functional assessment revealed that the genes modified by arsenite exposure were enriched for several biological functions, including cell cycle control, proteolytic enzymes, signal transduction, stress response, and transcriptional regulation. These findings indicated that iAs causes complex toxicopathological injury, as assessed at the transcriptional level (Yih et al., 2002).

A study performed in 2003 by Andrew et al., compared genomic responses resulting from acute exposure to low (5 M) versus high (50 M) levels of arsenic (as sodium arsenite) in human bronchial epithelial cells (Andrew et al., 2003). The two doses influenced the expression of almost completely distinct subsets of genes. The lower dose of arsenic exposure was shown to alter the expression levels of genes coding for various protein classes, including DNA repair proteins, inflammatory cytokines, kinases, and transcription factors. The higher dose of arsenic exposure increased the expression levels of genes coding for heatshock proteins. Genes involved in stress response pathways, and also jun kinases (JNKs) and

mitogen-activated protein kinase kinases (MAPPKs) were identified as increased at the expression level by the higher dose of arsenic. These metal response patterns may shed new light onto the mechanisms underlying arsenic-induced human disease. For example, they may be used in the development of biomarkers of exposure and/or effect in epidemiological, toxicological, and risk assessment studies (Andrew et al., 2003).

In 2003, Zheng et al., acutely exposed human embryonic kidney cells to 10 or 25 μM arsenite. A gene expression analysis showed that 20 genes were up-regulated and 19 genes were down-regulated at the expression level upon exposure to either arsenite exposure levels (Zheng et al., 2003). Of note, genes related to oxidative stress (e.g. *HMOX* or *HO-1*) and stress response were increased in expression, and proto-oncogenes (e.g. *MYC*, or V-myc avian myelocytomatosis viral oncogene homolog), chemokine receptors, and signaling molecules were decreased in expression by arsenite. Genes encoding proteolytic enzyme and transcription factors were also modified by arsenite. These findings showed that arsenic causes complex cellular injury and cell adaptation to arsenite is related to alterations in the expression levels of many different genes (Zheng et al., 2003).

Genes involved in arsenic-induced reactive oxygen species (ROS) production were identified by Chou et al., in 2005 (Chou et al., 2005). In addition to contributing to the production of arsenic-induced ROS, a set of genes were also hypothesized to be responsive to arsenic-induced ROS. To evaluate this hypothesis, genes were identified whose expression levels respond to arsenic and hydrogen peroxide and whose modified expression induced by arsenic was reversed by a ROS scavenger. Within the evaluated acute promyelocytic leukemia cells, 26 genes were identified as responsive to arsenic, 6 of which could be linked to ROS production by arsenic. Researchers also found that arsenic-induced oxidation of the Sp1 transcription factor (SP1) played a role in the suppressed gene expression induced by arsenic-associated ROS production. These findings showed that a fraction of the genes modified by arsenic are involved in ROS production and that ROS can suppress gene expression levels, in part through SP1 oxidation (Chou et al., 2005).

Kawata et al., employed microarray technology to classify the toxicities of multiple environmental toxicants and identify candidate toxicant-specific gene biomarkers using human hepatoma cells in 2007 (Kawata et al., 2007). The genomic responses to six heavy metals, namely antimony, arsenic, cadmium, chromium, mercury, and nickel were compared with responses induced by the model toxicants, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), phenol, and N-nitrosodimethylamine. Arsenic exposure, in addition to the other metals, was found to increase the expression levels of genes related to oxidative stress. Interestingly, the gene expression alterations and hierarchical clustering analysis showed that the biological action of the six heavy metals were closely related to that of DMNQ, an agent known to induce ROS. These results indicate that mechanistic-based classifications using DNA microarrays can be implemented to classify the toxicities of environmental agents (Kawata et al., 2007).

In 2009, Bourdonnay et al., exposed human blood monocyte-derived macrophages to low concentrations of arsenic trioxide and assessed the resulting genomic responses (Bourdonnay et al., 2009). Many of the genes that showed modified expression levels by arsenic exposure were related to the differentiation program of human macrophages. Some notable genes that were validated using gene-specific approaches included *CCL2* (chemokine (C-C motif) ligand 2), *CXCL2* (chemokine (C-X-C motif) ligand 2), *MMP9* (matrix metallopeptidase 9), *MMP12* (matrix metallopeptidase 12), and *SPON2* (spondin 2, extracellular matrix protein), which are

known to regulate major macrophagic functions. Performing an additional experiment with human alveolar macrophages, similar genomic responses to arsenic were found. The data support that environmentally relevant levels of arsenic impair important genes involved in the differentiation program of human macrophages (Bourdonnay et al., 2009).

Our laboratory carried out a study in 2010, where we investigated the anti-tumor properties of arsenic using a systems biology approach to identify gene biomarkers underlying tumor cell responses to arsenic (Liu et al., 2010). Using the NCI-60 tumor cell line panel, investigators identified a significant association between the baseline expression levels of 209 genes and the sensitivity of the cell line to arsenic-induced cytotoxicity. Networks that likely modulated the tumor cell responses to arsenic were identified as enriched for the oxidative stress response pathway mediated by NRF2. The role of the Nrf2 pathway in the protection of tumor cells against arsenic-induced cytotoxicity was validated *in vitro*, where tumor cells that were deficient for *NRF2* showed increased sensitivity to arsenic. The results of this study were highlighted to increase the understanding of mechanisms underlying arsenic-induced cytotoxicity, as well as increase the applicability of arsenic trioxide as a chemotherapeutic agent in cancer treatment (Liu et al., 2010).

Continuing this work with arsenic, we assessed the genomic response of human lymphoblast cells exposed to low-dose arsenic in 2011 (Benton et al., 2011). This study identified 62 genes with altered expression levels resulting from acute exposure to 0.1 μM sodium arsenite. A systems-level analysis of the transcriptional responses showed that arsenic modified signaling networks enriched for important biological processes, including antimicrobial response, cancer, cell death, cell development, cell signaling, and inflammation. Analysis of the promoter regions of the arsenic-modulated genes revealed an enrichment for transcriptional regulation by E2F transcription factor (E2F), octamer-1 transcription factor (OCT-1), and RBP-JKappa transcription factor. Networks constructed using the arsenic-associated genes contained pathways involving HNF-4, IL1, and TNF-α signaling. Also of note, when arsenic and cadmium-modulated gene sets were analyzed together at the systems biology-level, genes were mapped onto overlapping networks containing p38 MAPK and HNF-4 pathways. These findings show that even at low exposure levels, iAs can modify important cellular pathways (Benton et al., 2011).

A novel genome-wide functional RNA interference (RNAi) screening strategy was used in 2012 by Oh et al*.,* to identify genes mediating arsenic-induced endoplasmic reticulum (ER) stress (Oh et al., 2012). This type of stress is of interest, as adaptive responses to ER stress are involved in cancer and diabetes, and understanding events underlying arsenic-induced ER stress likely provides knowledge about disease development. A short hairpin RNA (shRNA) library targeting ~20,000 genes was used in conjunction with an ER stress model to identify genes modulating arsenite-induced ER stress. The investigators identified sodium-dependent neutral amino acid transporter (SNAT2) as a mediator of arsenic-induced ER stress and also suggested a role for disrupted mammalian target of rapamycin (mTOR) signaling in arsenic-induced disease. This study exemplifies how RNAi screens can be used to uncover mechanisms linking environmental exposures to disease (Oh et al., 2012).

Stueckle *et al.,* in 2012 set out to identify cancer promoting signaling networks associated with arsenic-transformed cells (Stueckle et al., 2012). In this study, human bronchial epithelial cells were exposed to low-dose arsenic trioxide. Cells were transformed after 6 months of exposure, showing significantly increased colony formation, proliferation, and invasion compared to unexposed cells. Gene expression analysis revealed the increased

expression of genes involved in mitochondrial metabolism and increased ROS protection in arsenic-transformed cells. A cancer-related network associated with the arsenic-modified genes was shown to include signaling related to anti-apoptosis, cell proliferation, inflammation, metabolism, and mobility. Networks also included Akt (v-akt murine thymoma viral oncogene homolog 1), EF1α (eukaryotic translation elongation factor 1 alpha 1), MAPK, and NFκB-related signaling suggested to promote genetic disorder, alterations in cell cycle, changes in metabolism, and cancer. This study showed that arsenic-transformed bronchial cells display disruptions in gene expression profiles that can be used for future lung cancer signaling research and possible arsenic exposure risk assessments (Stueckle et al., 2012).

3.1.2. Assessment of Inorganic Arsenic Using Rodent Models

Animal models have been used to evaluate the effects of arsenic exposure at the systems biology level. The Waalkes laboratory, in particular, has investigated the effects of arsenic exposure *in vivo* through several investigations. For example, Liu *et al.,* assessed the effects of acute arsenic exposure on stress-related gene expression in mice in 2001 (Liu et al., 2001). Mice received injections of either sodium arsenite (100 μmol/kg), sodium arsenate (300 μmol/kg), or saline. Three hours post-injection, livers were removed and assessed for stress-related gene expression using a mouse stress/toxicology array. The arsenic exposures altered the expression levels of genes associated with DNA damage, metabolism, and stress. Some notable genes that showed significant increases in expression resulting from arsenic exposure included *Ho-1* (heme oxygenase 1), *Hsp60* (heat shock protein-60), the DNA excision repair gene *Ercc1* (excision repair cross-complementing rodent repair deficiency, complementation group 1), and the DNA damage inducible gene *Gadd45*. Arsenic exposure was also found to activate the AP-1/C-JUN transcription complex. Proteins with arsenic-induced increased expression included NFκB, AP-1/C-JUN, and TNF-α. This study profiled the expression patterns of genes related to stress in mice exposed to inorganic arsenicals, contributing to the understanding of acute arsenic poisoning and toxicity (Liu et al., 2001).

The Waalkes research group performed another study in 2004, which evaluated disruptions in gene expression associated with transplacental arsenic hepatocarcinogenesis in mice (Liu et al., 2004). Using custom mouse cancer arrays, the expression profiles across 600 genes were analyzed in liver tumors and nontumorous livers from adult mice exposed to arsenic *in utero*. Liver tumors in arsenic-exposed mice had more genes that exhibited disrupted expression profiles (n=82) in comparison to liver tumors that spontaneously occurred in unexposed mice (n=46). In the arsenic-induced hepatocellular carcinoma samples, genes with altered expression included oncogenes, tumor suppressor genes, stress-related genes (e.g. *Ho-1* and *Hsp84*), and genes encoding for cell proliferation, hormone receptors, and metabolic enzymes. In arsenic-exposed mice that did not develop hepatocellular carcinoma, disruptions in the expression levels of 60 genes were still observed, reflecting arsenic-induced gene disruptions in preneoplastic stages. In these liver samples, arsenic was found to alter the expression levels of oncogenes, stress-related genes, genes encoding metabolic enzymes, and genes encoding for growth factors and cell communication. The aberrant liver gene expression profiles resulting from transplacental arsenic exposure were notable, as the expression changes were observed in adulthood, well after the gestational arsenic exposure period (Liu et al., 2004).

Continuing their research in 2004, the Waalkes laboratory exposed male mice to drinking water containing 45 ppm arsenic (as $NaAsO_2$) for 48 weeks (Chen et al., 2004). This long-

term exposure caused hepatic steatosis to occur in exposed mice. Microarrays were used to assess the expression levels of 588 genes in the liver, where 30 were identified with disrupted expression resulting from arsenic exposure. Genes that were affected by arsenic exposure included those related to apoptosis, cell cycle, steroids, and cytokines. The investigators also noted that estrogen receptor alpha (ERα) and cyclin D1 (CCND1) were increased at the protein and gene expression level. This long-term exposure to arsenic was concluded to disrupt the expression levels of important genes *in vivo* (Chen et al., 2004).

The Waalkes research group further explored the genomic impact of transplacental arsenic exposure-induced hepatocarcinogenesis in 2006 (Liu et al., 2006). In this study, pregnant mice were given drinking water containing 85 ppm sodium arsenite between days 8 to 18 of gestation. In the adult male mice offspring, hepatocellular carcinoma occurred at a 4-fold increased incidence in exposed-mice versus unexposed mice. A genome-wide assessment probing across 22,000 oligonucleotides within the liver samples revealed that *in utero* arsenic exposure altered the expression of 2,010 genes in arsenic-exposed normal liver samples compared to unexposed samples, and 2,540 genes in arsenic-induced hepatocellular carcinoma samples. Compared to their previous transplacental study that was based off a smaller gene analysis (Liu et al., 2004), the gene expression alterations were largely consistent. The arsenic-modulated genes included oncogenes and were also noted for their roles in cell communication, cell proliferation, and stress. There was an overexpression of ER-α-linked gene expression alongside increased levels of ER-α and ER-α-linked proteins in the livers of mice bearing arsenic-induced hepatocellular carcinoma. MYC activation was also identified as a likely player in transplacental arsenic carcinogenesis. Important signaling pathways were, therefore, associated with transplacental arsenic-induced hepatocarcinogenesis (Liu et al., 2006).

To further evaluate the molecular events associated with fetal onset of arsenic hepatocarcinogenesis, the same research group carried out another study in 2007 by Liu *et al* (Liu et al., 2007). Using a similar exposure paradigm as in the previous study, pregnant mice were exposed to arsenic-contaminated drinking water during gestation. Instead of evaluating livers from the adult offspring, this study evaluated the fetal livers at the end of the arsenic exposure period. Genome-wide expression analysis revealed that *in utero* exposure to arsenic significantly modified the expression levels of 187 genes within the fetal livers, 25% of which were related to either estrogen signaling or steroid metabolism. Methionine metabolism was also enriched for amongst the arsenic-modulated genes. Together, the results found by the Waalkes laboratory showed that iAs exposure during critical development periods in developing mouse fetuses alters the expression levels of genes related to estrogen signaling, metabolism, and stress, among others. These alterations may influence genetic programming at early life stages, resulting in possible tumor formation later in adulthood (Liu et al., 2007).

In 2007, Andrew et al., evaluated genome-wide transcriptional responses within the lung of mice exposed to various levels of iAs (Andrew et al., 2007). Mice were exposed at levels representative of drinking water wells within the U.S. (0, 0.1, or 1 ppb), in addition to a higher exposure level of 50 ppb for five weeks. Compared to the unexposed mice, mice exposed to 0.1 ppb arsenic showed 94 modified transcripts, mice exposed to 1 ppb arsenic showed 26 modified transcripts, and mice exposed to 50 ppb showed 37 modified transcripts. Arsenic was found to alter the expression levels of genes with roles in angiogenesis, apoptosis, cell cycle, immune response, lipid metabolism, and oxygen transport. A pathway map was constructed using the arsenic-modulated genes, where CCND1, MAPK1, SP1, and

P53 were identified as likely central regulators of the transcriptional response. These arsenic-associated signaling pathways were implicated to help guide thresholds for biological effects and aid in the risk assessment of arsenic-induced disease (Andrew et al., 2007).

Kozul et al., evaluated the effects of arsenic exposure through food/water consumption in the mouse lung in order to increase understanding of arsenic-induced lung disease in 2009 (Kozul et al., 2009). Mice were exposed to 10 or 100 ppb arsenic (as sodium arsenite) in drinking water or food for several weeks. Genome-wide transcriptional analysis of lung samples revealed that arsenic exposure disrupted gene expression profiles after both levels of exposure, where 10 ppb arsenic primarily down-regulated the expression of many transcripts. Animals exposed to arsenic within their food had gene expression alterations similar to those caused by drinking water exposure. Arsenic-modified genes were functionally related to cell adhesion and migration, channels, receptors, differentiation and proliferation, and innate immune response. A network analysis of the arsenic-associated genes revealed that both 10 ppb and 100 ppb arsenic exposure via contaminated drinking water disrupted signaling related to the inflammatory cytokine IL1β which was decreased at the gene expression level by both exposure levels. These results show that chronic exposure to iAs, even at low doses, can induce alterations in genes related to important signaling pathways that may contribute to disease, particularly in the lung (Kozul et al., 2009).

In 2011, Robinson et al., evaluated arsenic embryotoxicity using a mouse model (Robinson et al., 2011). Pregnant mice were injected with single doses of sodium arsenite or water on gestation day 8, a time when neurulation occurs. Within hours after the injection, mice were euthanized and the developing embryos were assessed for changes in genome-wide expression profiles. Dose-dependent gene expression changes associated with iAs exposure were identified and related to cell cycle, glutathione metabolism, response to UV, RNA processing, and alcohol, sugar, and sterol metabolism. This study quantitatively identified dose-dependent effects induced by iAs on gene expression patterns in mouse embryos during a window of embryotoxicity sensitivity (Robinson et al., 2011).

3.1.3. Assessment of Inorganic Arsenic in Humans

The impact of arsenic exposure in humans has been assessed at the systems biology level using populations across the world with known exposure to arsenic. For instance, in 2001, Lu *et al.,* assessed gene expression profiles within the livers of populations with arsenic-induced skin lesions and hepatomegaly in Guizhou, China (Lu et al., 2001). For a comparison group, normal human liver tissues were provided by hospitals within the U.S. from surgical resection or rejected transplants. Using microarrays which covered approximately 600 genes, a statistical comparison of transcript levels revealed that 60 genes were differentially expressed in arsenic-exposed liver tissue compared to normal human liver tissue. These arsenic-associated genes were identified as enriched for various biological processes, including apoptosis, cell cycle regulation, DNA damage response, and intermediate filaments. This initial human study using arrays was implicated to help formulate strategies to prevent and/or treat arsenic-induced liver injuries in humans (Lu et al., 2001).

In 2003, Wu et al., investigated potential transcripts involved in atherosclerosis that may result from prolonged exposure to arsenic (Wu et al., 2003). In this study, human lymphocytes were collected from Taiwanese subjects with varying levels of blood arsenic likely resulting from exposure to contaminated well water. A total of 62 genes were identified as differentially expressed in intermediate or high arsenic-exposed groups compared to low

arsenic-exposed groups. Many of the altered genes included cytokines and growth factors involved in inflammation, including chemokine C-C motif ligand 2/monocyte chemotactic protein-1 (*CCL2*/*MCP1*), which was confirmed as associated with arsenic exposure at the plasma protein level. These results indicated that the arsenic-associated increased expression levels of inflammatory molecules may act as contributing factors to atherosclerosis. Such multidisciplinary studies involving epidemiology will facilitate the uncovering of the role of arsenic-associated inflammation in atherosclerosis pathogenesis (Wu et al., 2003).

A population in Bangladesh exposed to arsenic-contaminated drinking water was investigated by Argos et al., in 2006 (Argos et al., 2006). Genome-wide transcriptional profiles were assessed in peripheral blood leukocytes of individuals with and without arsenical skin lesions. Comparing these two groups revealed that 312 genes were significantly differentially expressed when restricting the analysis to female never-smokers. A pathway enrichment analysis of these arsenic-associated genes was performed, where one pathway was identified that could discriminate between the subjects with and without skin lesions: signal transduction through IL1 receptor. This pathway included the following arsenic-associated differentially expressed genes: *IL1A*, *IL1B*, *IL6*, *JUN*, *MAP3K1*, *MAP3K7*, *MAPK14*, *NFKBIA*, *NFKB1*, and *TNF*, among others. The arsenic-associated genes may represent targets for chemoprevention studies to decrease arsenic-induced skin cancer (Argos et al., 2006).

Another study was performed using samples collected from a population in Bangladesh by Fry et al., in 2007 (Fry et al., 2007). This study investigated the genomic impact of prenatal exposure to arsenic, where gene expression profiles in the cord blood of newborns whose mothers were exposed to varying levels of arsenic during pregnancy were evaluated. A total of 447 genes were identified as differentially expressed in newborns whose mothers were exposed to arsenic in comparison to newborns whose mothers were unexposed. A gene ontology enrichment analysis revealed that these genes were involved in a variety of biological processes and functions, including cell death, cytokine activity, immune response, inflammatory response, and response to stress. An alternative enrichment analysis was performed, namely gene set enrichment analysis, where the arsenic-associated genes were identified as enriched for various signatures, including those related to hypoxia/stress, cancer, tumor promotion, and tumor progression. In order to further assess the effects of prenatal arsenic exposure at the mechanistic level, molecular networks were constructed using protein products of the arsenic-associated genes. These networks involved signaling related to the early growth response 1 (EGR-1), hypoxia inducible factor-1 α (HIF-1α), IL1β, JUNB, NFκB, signal transducer and activator of transcription (STAT1), and TNF-α. This study showed prenatal arsenic exposure heavily influences gene expression patterns and important signaling pathways in newborns (Fry et al., 2007).

In 2008, a study was performed by Andrew et al., using a U.S. population (Andrew et al., 2008). Peripheral blood leukocytes collected from individuals in New Hampshire were assessed for arsenic-associated gene expression changes. Comparing transcript levels of individuals with high versus low levels of arsenic exposure revealed that 259 genes were associated with exposure. These arsenic-associated genes were functionally related to apoptosis, cell growth, defense response, diabetes, immune function, cell cycle regulation, and the T cell receptor (TCR) signaling pathway. The high arsenic-exposed group showed higher levels of several killer cell immunoglobulin-like receptors that are known to inhibit natural killer cell activity. These transcriptional responses to chronic arsenic exposure provide

an increased understanding on arsenic-induced diseases, and may represent potential targets for monitoring arsenic exposure (Andrew et al., 2008).

3.1.4. Summary of Systems-level Findings for Inorganic Arsenic

Arsenic is a well-studied compound that has been assessed at the systems biology level using *in vitro* models, experimental animals, and humans. Many biological functions have been identified as associated with 'omic responses to arsenic across multiple studies (Table 7). Of the 26 studies summarized on arsenic, six studies showed that arsenic exposure is associated with altered apoptosis (Andrew et al., 2008; Andrew et al., 2007; Chen et al., 2001; Chen et al., 2004; Lu et al., 2001; Stueckle et al., 2012) and eight showed that arsenic is associated with altered cell cycle regulation at the systems biology level (Andrew et al., 2008; Andrew et al., 2007; Chen et al., 2001; Chen et al., 2004; Lu et al., 2001; Robinson et al., 2011; Stueckle et al., 2012; Yih et al., 2002). These findings are relevant to disease development, as impaired apoptosis and altered cell cycle regulation can lead to cellular transformation and carcinogenesis (Hanahan et al., 2011).

Various signaling pathways have also been associated with arsenic-induced responses (Table 8). Among these pathways is ERα signaling, which is distinctively responsive to arsenic exposure within the studies reviewed in this chapter. ERα is a ligand activated transcription factor that mediates a range of biological effects in the cardiovascular, central nervous, immune, and musculoskeletal systems (Heldring et al., 2007). ERα signaling can be disrupted in many different diseases, including cancer (Heldring et al., 2007) and diabetes (Tiano et al., 2011). As summarized in this review, iAs exposure has been shown to impact ERα signaling in rodents (Chen et al., 2004), even after *in utero* exposure (Liu et al., 2007). These changes induced *in utero* were shown to persist into adulthood in arsenic-induced tumors (Liu et al., 2006). Sp1 signaling is also a responder to arsenic based on the studies reviewed in this chapter. SP1 is an important transcription factor that interacts with the transcription initiation complex, histone modifying enzymes, and chromatin remodeling complexes (Li et al., 2010). Dysregulation of Sp1 signaling can occur in different diseases, including cancer (Li et al., 2010). These pathways may, therefore, play a role in arsenic-induced disease.

Table 7. Biological functions associated with exposure to arsenic. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Apoptosis	*in vitro*	Chen 2001, Stueckle 2012
	rodent	Chen 2004, Andrew 2007
	human	Lu 2001, Andrew 2008
Cancer	*in vitro*	Benton 2011, Stueckle 2012
	human	Fry 2007
Cell communication	rodent	Liu 2004, Liu 2006
Cell cycle regulation	*in vitro*	Chen 2001, Yih 2002, Stueckle 2012
	rodent	Chen 2004, Andrew 2007, Robinson 2011
	human	Lu 2001, Andrew 2008
Cell death	*in vitro*	Benton 2011
	human	Fry 2007
Cell differentiation	*in vitro*	Bourdonnay 2009
	rodent	Kozul 2009

Table 7. (Continued)

Enriched Function	*Model*	Reference(s)
Cell growth	*in vitro*	Simeonova 2000
	human	Andrew 2008
Cell proliferation	*in vitro*	Stueckle 2012
	rodent	Liu 2004, Liu 2006, Kozul 2009
Cytokine production	*in vitro*	Chen 2001, Andrew 2003
	rodent	Chen 2004
	human	Wu 2003, Fry 2007
DNA damage response/repair	*in vitro*	Bae 2002, Andrew 2003
	rodent	Liu 2001
	human	Lu 2001
Growth-factor production	*in vitro*	Chen 2001
	rodent	Liu 2004
	human	Wu 2003
Heatshock protein production	*in vitro*	Andrew 2003
	rodent	Liu 2001
Hormone-receptor production	*in vitro*	Chen 2001
	rodent	Liu 2004
Inflammation	*in vitro*	Andrew 2003, Benton 2011, Stueckle 2012
	human	Wu 2003, Fry 2007
Immune function/response	rodent	Andrew 2007, Kozul 2009
	human	Fry 2007, Andrew 2008
Metabolism	*in vitro*	Stueckle 2012
	rodent	Liu 2001, Liu 2004, Liu 2007, Andrew 2007, Robinson 2011
Oxidative stress	*in vitro*	Zheng 2003, Chou 2005, Kawata 2007, Liu 2010
Proteolytic enzyme production	*in vitro*	Yih 2002, Zheng 2003
Signal transduction	*in vitro*	Chen 2001, Yih 2002
Stress response	*in vitro*	Chen 2001, Yih 2002, Andrew 2003, Zheng 2003
	rodent	Liu 2001, Liu 2004, Liu 2006
	human	Fry 2007
Transcription factor production	*in vitro*	Andrew 2003, Zheng 2003
Transcriptional regulation	*in vitro*	Yih 2002, Benton 2011
	rodent	Andrew 2007

Table 8. Pathways associated with exposure to arsenic

Enriched Pathway	Model	Reference(s)
Akt signaling	*in vitro*	Stueckle 2012
AP-1/c-Jun signaling	*in vitro*	Simeonova 2000
	rodent	Simeonova 2000, Liu 2001
CCND1 signaling	rodent	Chen 2004, Andrew 2007
EF1α signaling	*in vitro*	Stueckle 2012
EGR-1 signaling	human	Fry 2007
ERα signaling	rodent	Chen 2004, Liu 2006, Liu 2007
HIF-1alpha signaling	human	Fry 2007
HNF-4 signaling	*in vitro*	Benton 2011
IL1 signaling	*in vitro*	Benton 2011
	rodent	Kozul 2009

Enriched Pathway	Model	Reference(s)
	human	Argos 2006, Fry 2007
MAPK signaling	*in vitro*	Andrew 2003, Benton 2011, Stueckle 2012
	rodent	Andrew 2007
mTOR signaling	*in vitro*	Oh 2012
MYC signaling	rodent	Liu 2006
NFkB signaling	*in vitro*	Stueckle 2013
	rodent	Liu 2001
	human	Fry 2007
Nrf2 signaling	*in vitro*	Liu 2010
p53 signaling	rodent	Andrew 2007
Sp1 signaling	*in vitro*	Chou 2005
	rodent	Andrew 2007
STAT1 signaling	human	Fry 2007
TCR signaling	human	Andrew 2008
TNF-α signaling	*in vitro*	Benton 2011
	rodent	Liu 2001
	human	Fry 2007

3.2. Cadmium

Cadmium is a known human carcinogen currently ranked #7 by the ATSDR (ATSDR 2011). It is a rare element mainly recovered as a by-product of zinc concentrates (IARC 2012c; NTP 2011). Cadmium exhibits chemical properties that are well suited for a wide variety of industrial applications. For example, cadmium is used in nickel-cadmium batteries, pigments, coatings and platings, stabilizers for plastics, and lubricants. Cadmium was also historically used as a fungicide for golf courses and home lawns, but these uses were banned in the late 1980s (IARC 2012c; NTP 2011).

The general population is exposed to cadmium and cadmium compounds primarily though the consumption of food and drinking water (NTP 2011). Other sources of exposure include inhalation of particles containing cadmium within the ambient air or cigarette smoke and ingestion of soil and dust contaminated with cadmium (NTP 2011). Occupational exposure to cadmium can also occur for workers involved in smelting zinc and lead ores, working with solders that contain cadmium, welding or remelting cadmium-coated steel, and producing/handling cadmium powders (NTP 2011).

In human populations, cadmium and cadmium compounds are known to cause lung cancer, and cadmium exposure has also been associated with cancer of the kidney and prostate (IARC 2012c; NTP 2011). In experimental animals, cadmium exposure has been found to cause leukemia, lymphoma, and tumor formation within the adrenal-glands, liver, lung, pituitary-glands, prostate, and testicles (NTP 2011). Other health effects linked to cadmium exposure in humans include diabetes, diabetic nephropathy, decreased lung function, hypertension, myocardial infarction, periodontal disease, peripheral artery disease, and age-related macular degeneration (Satarug et al., 2010). Because cadmium exposure can cause a variety of adverse health effects, it is important to understand the mechanistic impact of cadmium exposure at the systems-level.

3.2.1. In Vitro Assessment of Cadmium

Cadmium exposure has been evaluated at the systems-level through the use of *in vitro* models. For instance, in 2002, Yamada and Koizumi assessed the transcriptional response of HeLa cells exposed to a non-lethal, low dose of cadmium (as $CdSO_4$) (Yamada et al., 2002). An array was used to probe the expression levels across 7,075 genes, where 46 genes were identified with cadmium-induced increased expression and 10 genes with cadmium-induced decreased expression. Many of the genes with increased expression included those encoding for metallothioneins and heat shock proteins, which are heavily involved in stress response. Changes in the expression levels of genes related to cell metabolism and antioxidant responses were also identified, along with activation of the ubiquitin pathway. The data support that human cells mobilize a variety of genomic resources to overcome cytotoxicity caused by cadmium exposure (Yamada et al., 2002).

Koizumi and Yamada continued their work in 2003 by publishing a review that included new results from a study assessing transcriptomic effects induced by a high dose (50 μM) of cadmium in HeLa cells (Koizumi et al., 2003). In comparison to the previous study using a lower cadmium dose, more genes displayed altered expression levels after the high dose of cadmium. More specifically, of the 9,182 genes assessed, 82 were identified with increased expression and 75 with decreased expression in response to cadmium. Genes affected by the high dose of cadmium exposure were related to apoptosis, cell protection, growth inhibition, protein degradation, and transcriptional activation. These changes in gene expression were implicated to serve as important clues to understand cadmium response-related events (Koizumi et al., 2003).

In 2005, Tan et al., exposed primary rat hepatocytes to cadmium acetate at varying doses (Tan et al., 2006). Gene expression profiles were assessed at multiple times post-exposure. Gene expression levels were identified to correlate with exposure-induced cytotoxicity. These gene groups were noted to include genes coding for DNA repair enzymes, heat shock proteins, phase I and phase II metabolizing enzymes, and MAPKs. Using the genes associated with cadmium-induced cytotoxicity, a hypothetic network was constructed to illustrate how cells respond to cadmium-induced stress. This network included signaling related to apoptosis, oxidative stress, MAPK, MYC, JUN, and TNF. The results from this study revealed candidate biomarkers of cadmium exposure through bioinformatics and high-throughput data analysis in order to aid in the understanding of molecular mechanisms underlying cadmium-induced toxicity (Tan et al., 2006).

As previously described, Kawata et al., classified the toxicities of multiple environmental toxicants and identified candidate toxicant-specific gene biomarkers using transcriptional responses in 2007 (Kawata et al., 2007). The genomic responses to six heavy metals, namely arsenic, cadmium, nickel, antimony, mercury, and chromium, were compared with responses induced by the model toxicants, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), phenol, and N-nitrosodimethylamine in human hepatoma cells. Similar to the other metals, a 2 μM exposure to cadmium (as cadmium chloride) was found to increase the expression levels of genes related to oxidative stress. Further supporting cadmium's association to oxidative stress, the gene expression alterations and hierarchical clustering analysis showed that the biological action of all six heavy metals were closely related to that of DMNQ, an agent known to induce ROS (Kawata et al., 2007).

The relationship between cadmium exposure, cell viability, and gene expression was evaluated in rat primary hepatocytes by Badisa et al., in 2008 (Badisa et al., 2008). The

expression profiles of 207 stress-related genes were evaluated in cells exposed to 100 μM cadmium (as cadmium chloride) across three treatment durations. Each treatment period caused different patterns of altered gene expression to occur. To detail, after 4 hours of cadmium exposure, three genes were increased and six genes were decreased in expression. A longer, 8 hour exposure to cadmium resulted in the increased expression of six genes and decreased expression of 14 genes. After 24 hours of cadmium exposure, 15 genes were decreased and six were increased in expression. The genes disrupted by cadmium included those coding for heat shock proteins and genes involved in cell cycle regulation, stress response, and basic cell function. The cells maintained viability for up to 8 hours of cadmium exposure putatively because of the expression of heat shock proteins and stress response proteins. The longer exposure caused cell toxicity and cell death likely from the down-regulation of basic cell function proteins and cell cycle regulation proteins (Badisa et al., 2008).

In 2010, Yu et al., investigated transcriptional responses of mouse embryonic fibroblast cells treated with cadmium (Yu et al., 2010). A 24 hour exposure to 5 μM cadmium was found to increase the expression levels of many genes, including those enriched for several biological processes (e.g. response to stimulus, regulation of protein kinase activity, transport, and ubiquitin-dependent protein catabolism) and molecular processes (e.g. heat shock protein activity and transporter activity). Cadmium was found to decrease the expression levels of genes involved in cell cycle, DNA metabolism, response to stimulus, immune response, oxidoreductase activity, and metallopeptidase activity. Comparing the cadmium-altered gene expression patterns to those altered by a classical proteasome inhibitor, MG132, revealed that both treatments altered some common gene groups. These included modulators of the ubiquitin-proteasome system, antioxidant and phase II detoxifying enzyme genes, and genes involved in cell cycle regulation pathways. The investigators related these findings to Parkinson's disease pathogenesis, suggesting that the cadmium-disrupted pathways may play roles in the development of diseases associated with metal exposure (Yu et al., 2010).

As previously noted, a study performed by Benton et al., in 2011 examined the systems-level effects of low-dose cadmium exposure in human TK6 lymphoblastoid cells (Benton et al., 2011). A genome-wide transcriptional assessment revealed that 0.1 μM cadmium (as cadmium chloride) exposure altered the expression levels of 105 genes. These cadmium-associated genes were functionally enriched for several biological processes and diseases, including cancer, cellular compromise, cell cycle regulation, cardiovascular disease, and carbohydrate metabolism. Cadmium-modulated networks were constructed, which contained signaling associated with NFκB, MYC, p38 MAPK, and p53. Transcription factors that were predicted to regulate the cadmium-altered genes included SP1, SREBP-1 (sterol regulatory element-binding protein-1), and ZF5 (zinc finger protein 161 homolog). These results show that even at low levels, cadmium can modify genes with a diverse set of functions and associations to important cellular pathways (Benton et al., 2011).

The transcriptional responses to cadmium, chromium, and nickel in rat-liver derived cell lines were compared by Permenter et al., in 2011 (Permenter et al., 2011). Both common and distinct transcriptional groups were identified as altered by the three metals. Cadmium was found to modulate transcripts represented by 288 probesets in an array. These modulated transcripts were related to apoptosis, cell cycle regulation, oxidative stress, and multiple canonical pathways, including acute phase response, ATM, IL1, Nrf2, p53 and retinoic acid signaling. Of the 288 cadmium-associated probesets, 102 overlapped with probesets

associated with chromium and/or nickel. Cadmium, chromium, and nickel also shared an overall enrichment for oxidative response genes, influenced by NRF2-mediated stress response pathways. These findings provide evidence to suggest that cadmium, chromium, and nickel induce common effects when broadly viewed, but some differences exist between the modulated gene sets and mechanisms underlying toxicity (Permenter et al., 2011).

In 2012, Fabbri et al., investigated the transcriptional responses of human hepatoma cells exposed to 2 and 10 μM cadmium. The lower cadmium exposure was found to alter the expression levels of multiple genes belonging to the metallothionein family. The higher cadmium exposure was found to modulate the expression levels of a greater number of genes. Genes at increased expression from this exposure were associated with pathways related to cancer (e.g. the focal adhesion pathway) and inflammation, while genes at decreased expression were associated with pathways regulating liver function, including fatty acid metabolism. The results were detailed to provide an increased understanding of the complex mechanisms underlying the effects of cadmium, a carcinogenic metal (Fabbri et al., 2012).

3.2.2. Assessment of Cadmium Using Rodent Models

The systems-level effects of cadmium exposure have been investigated to an extent in vivo using rodent models. For instance, in 2002, Liu et al., compared transcriptional responses to cadmium exposure within the liver of wild-type versus metallothionein-null mice (Liu et al., 2002). In both types of mice, cadmium exposure was found to increase the expression levels of genes involved in stress response (e.g. heat shock proteins), free radical production, and DNA damage response/repair. Cadmium exposure was also found to decrease the expression levels of genes encoding cytochrome P450 enzyme, among others. Metallothionein-null mice were identified as more sensitive than wild-type mice to changes in stress-related gene expression caused by cadmium exposure. MAP kinases were implicated in the transcriptional responses to cadmium exposure, where JNK1 and JNK2 phosphorylation was increased in both mice after cadmium exposure. AP-1 was also activated upon exposure to cadmium. These results suggest that the mechanism underlying cadmium-induced toxicity involves multiple facets, including aberrant gene expression and oxidative stress, and the absence of metallothionein exacerbates the influence of cadmium exposure on the transcriptome (Liu et al., 2002).

In 2004, Zhou et al., investigated the effects of low dose cadmium exposure on the rodent testes (Zhou et al., 2004). Mice were exposed with a single injection of 5 μmol/kg cadmium (as cadmium chloride), and transcriptional profiles were assessed 12-72 hours after exposure. Although the tested dose did not produce overt histopathological changes, cadmium exposure caused modifications in gene expression profiles that were often time-dependent. The most significant changes in transcript levels were found 24 hours after exposure, corresponding to when the highest level of cadmium was measured in the testes. Cadmium exposure was found to increase the expression levels of genes involved in acute stress response and DNA repair, decrease the expression levels of genes involved in apoptosis and cell cycle regulation, and alter the expression levels of genes involved in cell proliferation. These findings show that gene expression alterations may occur well before overt effects of cadmium exposure, including testicular toxicity and cancer development (Zhou et al., 2004).

A toxicogenomic study was performed by Robinson et al., in 2009 to compare cadmium-induced gene expression changes in developing embryos from two different mouse strains, C57BL/6 (C57) and SWV (Robinson et al., 2009). In both mouse strains, cadmium was found

to alter the expression levels of genes involved in cell cycle regulation. In the C57 mouse strain, P53-dependent mediators were identified as up-regulated, which are known to influence apoptosis and neural tube defect formation. A greater cadmium-induced reduction in the expression levels of nervous system development-related genes was also identified in this strain of mouse. The investigators concluded that differences in cadmium-induced gene expression alterations exist between resistant and sensitive strains of mice (Robinson et al., 2009).

This research group expanded on their findings in 2011, where a previously mentioned study was performed to increase understanding on metal-induced embryotoxicity (Robinson et al., 2011). For this study, mouse embryos were exposed to various doses of cadmium during neurulation. A toxicogenomic dose-response relationship was identified, where genes involved in cell cycle, response to UV, glutathione metabolism, and RNA processing were associated with cadmium dose. Transcription factors predicted to regulate the observed transcriptional responses to metal exposure included FOXA2, NFκB1, and NRF1. These investigators quantitatively identified dose-dependent effects induced by cadmium on gene expression patterns in mouse embryos during a window of embryotoxicity sensitivity (Robinson et al., 2011).

In 2012, Ali et al., assessed the livers of male mice exposed to cadmium at various doses. Cadmium exposure was found to cause changes in kinase phosphorylation and gene expression profiles. Dose-response relationships were identified for the expression levels of metallothionein genes and stress response genes. Cadmium exposure was also found to increase activation of ERF1/2, P38 MAPK, and P53. These data suggest that *in vivo* effects on transcriptional profiles and cell signaling caused by cadmium are markedly dependent on concentration (Ali et al., 2012).

3.2.3. Summary of Systems-level Findings for Cadmium

Cadmium exposure-induced effects at the systems biology level have been investigated using mammalian cell culture and rodent models. Throughout the reviewed studies, many alterations in biological functions that were associated with cadmium exposure were consistently identified in two or more studies (Table 9). One persistent theme throughout the cadmium-related findings is the involvement of the metallothionein family in response to exposure (Ali et al., 2012; Fabbri et al., 2012; Yamada et al., 2002). Metallothionein proteins are high affinity metal binding proteins that commonly form complexes with cadmium within the body (Thévenod 2009). A model of metallothionein-null mice has even been used to evaluate changes in the transcriptome induced by cadmium, where metallothionein-null mice were found to experience more changes in gene expression profiles in comparison to wild-type mice, particularly in regards to stress-related genes (Liu et al., 2002). These findings provide strong evidence for metallothionein acting as a major player in cadmium exposure response.

Certain pathways were also identified as associated with cadmium exposure in the reviewed studies (Table 10). Of note, two of the 14 summarized studies found an association between cadmium exposure and ubiquitin signaling (Yamada et al., 2002; Yu et al., 2010). Ubiquitin was originally recognized for its role in tagging proteins for degradation through the proteasomal degradation pathway (Kirkin et al., 2007). More recently, ubiquitin signaling has become recognized as an important mediator of a diverse range of cellular processes, including cell cycle progression, endocytosis, DNA repair, receptor trafficking, and

transcription (Kirkin et al., 2007). Given the versatility of ubiquitin signaling, this pathway is receiving attention for its potential roles in disease pathogenesis and may contribute to cadmium-induced health effects.

Table 9. Biological functions associated with exposure to cadmium. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Antioxidant response	*in vitro*	Yamada 2002, Yu 2010
Apoptosis	*in vitro*	Koizumi 2003, Permenter 2011
	rodent	Zhou 2004, Robinson 2009
Cancer	*in vitro*	Benton 2011, Fabbri 2012
	rodent	Zhou 2004
Cell cycle regulation	*in vitro*	Basida 2008, Yu 2010, Benton 2011, Permenter 2011
	rodent	Zhou 2004, Robinson 2009, Robinson 2011
DNA damage response/repair	*in vitro*	Tan 2006
	rodent	Liu 2002, Zhou 2004
Heat shock protein activity/production	*in vitro*	Yamada 2002, Tan 2006, Basida 2008, Yu 2010
	rodent	Liu 2002
Metabolism	*in vitro*	Yamada 2002, Tan 2006, Benton 2011
	rodent	Robinson 2011
Metallothionein production	*in vitro*	Yamada 2002, Fabbri 2012
	rodent	Ali 2012
Oxidative stress	*in vitro*	Kawata 2007, Permenter 2011
Stress response	*in vitro*	Yamada 2002, Basida 2008
	rodent	Liu 2002, Ali 2012

3.3. Chromium

Chromium is currently ranked 17th on the ATSDR Priority List of Hazardous Substances (ATSDR 2011). Chromium is a naturally-occurring element present in animals, plants, rocks, and soil, where it commonly exists in combination with other elements (ATSDR 2012). Chromium exists as three main forms: chromium(0), chromium(III), and chromium(VI) (ATSDR 2012), where chromium(VI) compounds are known to be human carcinogens (NTP 2011). Chromium is released into the air, water, and soil from industries that use chromium, such as those involved in electroplating, leather tanning, textile production, and the manufacturing of chromium-based products, including steel. Chromium can also enter the air as a result of burning natural gas, oil, or coal (ATSDR 2012).

The general population can be exposed to chromium(VI) compounds through inhalation of ambient air contaminated with chromium, ingestion of chromium-containing water, or dermal contact with products containing chromium, including pressure-treated wood products (NTP 2011). Higher chromium(VI) exposures can occur in occupational settings, such as those related to stainless-steel welding, chromate production, chrome plating, ferrochrome alloy production, and chromate pigment production (NTP 2011). Several detrimental health

effects can result from chromium exposure. For instance, occupational exposure to chromium has been associated with allergic contact dermatitis and respiratory tract problems, including asthma, cough, shortness of breath, and wheezing (ATSDR 2012). It is well-established through experimental animal studies and epidemiological studies that inhalation exposure to chromium(VI) causes an increased risk of lung cancer (NTP 2011). Because chromium is a prevalent environmental exposure, coupled with its serious health effects, it is critical to understand the molecular pathways disrupted by chromium that potentially contribute to disease.

Table 10. Pathways associated with exposure to cadmium

Enriched Pathway	Model	Reference(s)
Acute phase response signaling	*in vitro*	Permenter 2011
	rodent	Zhou 2004
AP-1/c-Jun signaling	rodent	Liu 2002
	in vitro	Tan 2006
ATM signaling	*in vitro*	Permenter 2011
Focal adhesion pathway	*in vitro*	Fabbri 2012
IL1 signaling	*in vitro*	Permenter 2011
MAPK signaling	*in vitro*	Tan 2006, Benton 2011
	rodent	Liu 2002, Ali 2012
MYC signaling	*in vitro*	Tan 2006, Benton 2011
NFkB signaling	*in vitro*	Benton 2011
	rodent	Robinson 2011
Nrf2 signaling	*in vitro*	Permenter 2011
p53 signaling	*in vitro*	Benton 2011, Permenter 2011
	rodent	Robinson 2009, Ali 2012
Retinoic acid signaling	*in vitro*	Permenter 2011
TNF-α signaling	*in vitro*	Tan 2006
Ubiquitin signaling	*in vitro*	Yamada 2002, Yu 2010

3.3.1. In Vitro Assessment of Chromium

The effects of chromium exposure at the systems-level have been evaluated through the use of *in vitro* models. For example, in 2001, Ye and Shi investigated the transcriptional effects of *in vitro* exposure to chromium(VI) using human lung epithelial cells (Ye et al., 2001). Cells were treated for 2 hours with 300 μM potassium dichromate, and transcriptional profiles were assessed using high-density oligonucleotide arrays representing 2400 genes. The expression levels of 150 genes were identified as increased by chromium exposure, while the expression levels of 70 genes were identified as decreased by chromium exposure. Genes that were differentially expressed upon exposure to chromium were enriched for various functions, including calcium mobilization, cell cycle regulation, energy metabolism, protein synthesis, redox stress, and carcinogenesis. These findings provided new insight into the understanding on molecular mechanisms of the biological activities of chromium(VI) (Ye et al., 2001).

The effects of chromium(III) were evaluated by Cheng et al., in 2002 (Cheng et al., 2002). Although this form of chromium is non-carcinogenic in most biological systems, a

potential effect induced by chromium(III) in sperm was postulated. To test this possibility, mouse TM4 Sertoli-like cultured cells were exposed to a non-toxic dose of chromium(III) chloride for seven days. Microarray analysis revealed that chromium(III) induced differential expression of many genes, where the changes were considered modest with a range of 1.5 to 2.3-fold difference. After additional stringent statistical filters, 52 genes were identified as differentially expressed by chromium exposure. The chromium-modulated genes included those involved in cell defense, DNA damage, and ROS production, as well as genes coding for transcription factors (e.g. BTB and CNC homology 2 (*Bach2*)). These results indicate that, although chromium(III) is non-carcinogenic in most biological systems, it still significantly modifies gene expression profiles *in vitro* (Cheng et al., 2002).

In 2004, Wei et al., investigated the transcriptional response of mouse hepatoma Hepa-1 cells treated with benzo[a]pyrene and then chromium(VI) (Wei et al., 2004). Chromium was found to inhibit many benzo[a]pyrene-inducible genes, including those involved in various signaling transduction pathways, such as apoptosis, calcium-dependent regulation, cell cycle regulation, cell differentiation, drug metabolism, receptor-associated kinases, and transcriptional regulation. It was evident that the transcriptional inhibitory effect of chromium was both pronounced and well generalized, influencing a variety of cellular processes (Wei et al., 2004).

Gavin et al., investigated in 2007 the effects of chromium(VI) exposure using peripheral blood mononuclear cells collected from human volunteers of various ethnic backgrounds (Gavin et al., 2007). Once cultured, these cells were exposed to various concentrations of chromium(VI), and the expression of many cytokines and chemokines were identified as concentration-dependent. Chromium-responsive genes included those involved in apoptosis, cell cycle, intracellular signaling, immune response, inflammatory response, organelle organization and biogenesis, and RNA transport and binding. Several molecular pathways were also enriched amongst the chromium-altered genes, including fructose and mannose metabolism, antigen processing and presentation, natural killer cell mediated cytotoxicity, TCR signaling, leukocyte transendothelial migration, and endoplasmic reticulum-associated degradation pathway. These results were implicated to facilitate the identification and monitoring of effective treatments for maladies induced by chromium exposure (Gavin et al., 2007).

As previously detailed, Kawata et al., classified the toxicities of multiple environmental exposures and identified candidate toxicant-specific gene biomarkers using transcriptional responses in 2007 (Kawata et al., 2007). The genomic responses to six heavy metals, namely arsenic, cadmium, nickel, antimony, mercury, and chromium, were compared with responses induced by the model toxicants, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), phenol, and N-nitrosodimethylamine in human hepatoma cells. Similar to the other metals, a 20 μM exposure to potassium dichromate was found to increase the expression levels of genes related to oxidative stress. Further supporting chromium's association to oxidative stress, the biological action of all six heavy metals were closely related to that of DMNQ, an agent known to induce ROS (Kawata et al., 2007).

A subcellular proteomics-based analysis was carried out by Lei et al., in 2008 to assess the influence chromium(VI) exposure has on cell signaling with lung epithelial cells (Lei et al., 2008). Using two-dimensional gel electrophoresis and mass spectroscopy-based techniques, more than 30 proteins were identified with altered expression levels upon exposure to 10 μM potassium dichromate. Most of the chromium-associated proteins were

noted as involved in antioxidative treatment, reactive oxygen species-elicited responses, and induction of the p53 pathway. The investigators concluded that these results provide evidence at the protein level to support that chromium induces apoptosis of lung cells via ROS generation. This mechanism is likely involved in chromium-induced toxicity and carcinogenesis (Lei et al., 2008).

The impact of using chromium(VI) in metallic implants was evaluated *in vitro* at the proteomic level by Raghunathan et al., in 2010 (Raghunathan et al., 2010). Cultured osteoblasts and monocytes were exposed to chromium(VI) for a period of three weeks at concentrations that have been measured in patients with metal implants. Using two-dimensional gel electrophoresis, time-dependent changes in proteins levels were observed for cytoskeletal, glycolytic, and stress-related proteins. Proteins that were altered in expression by chromium were noted for their roles in cell function, including cell signaling, energy metabolism, and proliferation. The results from this study may be used to establish clinical biomarkers to monitor long-term use of metallic implants (Raghunathan et al., 2010).

As previously described, in 2011 Permenter et al., compared transcriptional responses to cadmium, chromium, and nickel exposures in rat liver-derived cell lines (Permenter et al., 2011). Both common and distinct transcriptional groups were identified as altered by the three metals. Chromium was found to modulate 456 probesets represented by an array, many of which were involved in cell cycle, DNA damage, oxidative stress, and multiple canonical pathways, acute phase response and maturity onset of diabetes signaling. Of the 456 chromium-associated probesets, 115 overlapped with probesets associated with cadmium and/or nickel. Cadmium, chromium, and nickel also shared an overall enrichment for oxidative response genes, influenced by NRF2-mediated stress response pathways. These findings suggest that cadmium, chromium, and nickel may induce common effects when broadly viewed, but some differences still exist between the modulated gene sets and mechanisms underlying toxicity (Permenter et al., 2011).

3.3.2. Assessment of Chromium Using Rodent Models

Chromium exposure has been assessed using rodent models in a few studies. In 2012, Kopec et al., investigated the effects of chronic exposure to chromium(VI) in mice. In this study, mice were exposed to various doses of sodium dichromate dehydrate through drinking water for 7 or 90 days, and genome-wide transcriptional profiles were assessed within the duodenal and jejunal epithelium. After 7 days of exposure, chromium was found to induce differential expression of 6562 genes within the duodenum and 4448 genes within the jejunum. After 90 days of exposure, chromium was found to induce differential expression of 4630 genes within the duodenum and 4845 genes within the jejunum. Many of the chromium-responsive genes overlapped between the two tissues, and these genes were enriched for various functions, including cell cycle regulation, immune response, lipid metabolism, and oxidative stress. Genes that were identified as dose-dependent included those regulated by NRF2. These findings were consistent with a chromium cancer mode of action involving oxidative stress and cytotoxicity as early key events (Kopec et al., 2012).

Also in 2012, Pan et al., investigated proteomic responses within the liver of mice dermally exposed to chromium (Pan et al., 2012). Comparing chromium(VI) versus chromium(III) dermal exposures, chromium(VI) was found to generate more oxidative stress, apoptosis, and hepatotoxicity. The assessment of over 1000 proteins using two-dimensional gel electrophoresis revealed that the levels of 25 proteins were modified by exposure to

chromium(VI) and chromium(III). The chromium-associated proteins were noted for their involvement in apoptosis, calcium homeostasis, carbohydrate metabolism, and endoplasmic reticulum (ER) stress. These findings provide evidence of chromium-induced hepatic damage and protein expression modifications through dermal exposure (Pan et al., 2012).

3.3.3. Summary of Systems-Level Findings for Chromium

A number of studies have assessed the effects of chromium exposure at the systems-level. Many biological functions have been associated with systems-level responses to chromium exposure (Table 11). Similar to arsenic, benzene, cadmium, cigarette smoke, and formaldehyde, chromium exposure was found to modify cell signaling related to apoptosis *in vitro* (Gavin et al., 2007; Wei et al., 2004) and *in vivo* (Pan et al., 2012). As previously mentioned, altered cellular apoptosis can cause severe consequences, including cellular transformation and tumor development (Hanahan et al., 2011).

Chromium exposure was also found to influence certain cell pathways (Table 12). Among these pathways is p53 signaling, which mediates the chromium-associated functions, apoptosis and cell cycle regulation. In particular, p53 signaling can initiate cell cycle arrest, DNA repair, senescence, and apoptosis, all of which contribute to a cell's capability of suppressing tumor formation (Vazquez et al., 2008). These altered pathways and cell functions may contribute to the pathogenesis of chromium-induced disease.

Table 11. Biological functions associated with exposure to chromium. Functions are listed for those identified by at least two studies

Enriched Function	Model	Reference(s)
Apoptosis	*in vitro*	Wei 2004, Gavin 2007
	rodent	Pan 2012
Cell cycle regulation	*in vitro*	Ye 2001, Wei 2004, Gavin 2007, Permenter 2011
	rodent	Kopec 2012
DNA damage response	*in vitro*	Cheng 2002, Permenter 2011
Endoplasmic reticulum stress	*in vitro*	Gavin 2007
	rodent	Pan 2012
Immune response	*in vitro*	Gavin 2007
	rodent	Kopec 2012
Metabolism	*in vitro*	Gavin 2007, Raghunathan 2010
	rodent	Kopec 2012
Oxidative stress	*in vitro*	Kawata 2007, Permenter 2011
	rodent	Kopec 2012
Reactive oxygen species production	*in vitro*	Cheng 2002, Lei 2007
Transcriptional regulation	*in vitro*	Cheng 2002, Wei 2004

Table 12. Pathways associated with exposure to chromium

Enriched Pathway	Model	Reference(s)
Natural killer cell signaling	*in vitro*	Gavin 2007
p53 signaling	*in vitro*	Lei 2008
TCR signaling	*in vitro*	Gavin 2007

4. Common Pathway Responses to Environmental Toxicants

Summarized within this review are many biological pathways altered by exposure to arsenic, benzene, cadmium, chromium, cigarette smoke, and/or formaldehyde. Comparing these reveals 12 toxicant-responsive pathways that are commonly altered by at least two contaminants: AP-1/c-Jun, EGFR, IL1, MAPK, MYC, natural killer cell, NFκB, Nrf2, p53, TCR, TGF-β, and TNFα signaling (Table 13).

Table 13. Pathways commonly altered by environmental exposures

Pathway Altered by at least Two Toxicants	Associated Toxicants
AP-1/c-Jun signaling	arsenic, cadmium
EGFR signaling	cigarette smoke, formaldehyde
IL1 signaling	arsenic, cadmium
MAPK signaling	arsenic, benzene, cadmium, cigarette smoke
MYC signaling	arsenic, cadmium
Natural killer cell signaling	chromium, cigarette smoke
NFκB signaling	arsenic, cadmium, formaldehyde
Nrf2 signaling	arsenic, cadmium, cigarette smoke
p53 signaling	arsenic, benzene, cadmium, chromium, cigarette smoke
TCR signaling	arsenic, benzene, chromium
TGF-β signaling	cigarette smoke, formaldehyde
TNF-α signaling	arsenic, cadmium

We hypothesized that the signaling pathways likely overlap and respond as a common sensor to environmental exposures in mammals. To test this potential overlap, the main proteins represented in each pathway identified throughout this review were overlaid onto a global interaction network. This included 11 of the 12 pathways excluding natural killer cell signaling. Networks containing these proteins were algorithmically constructed based on connectivity, as enabled through Ingenuity Pathway Analysis (Ingenuity Systems®, Redwood City, CA). Focusing on the proteins that directly interact within the toxicant-responsive pathway, a significant ($p<10^{-2}$) network was identified. This network illustrates trans-pathway interactions related to environmental exposures, termed the "Environmental Toxicant Signalisome" (Figure 1).

The Environmental Toxicant Signalisome represents pathways that are common responders to the environmental exposures assessed in this review. It provides evidence that there may be overlap in the systems-level responses to toxicants resulting from protein-protein connections between pathways. Such interactions may cause signaling to occur in a semi-concerted manner. This view is supported by the mapped connections between toxicant-responsive pathway mediators (Figure 1).

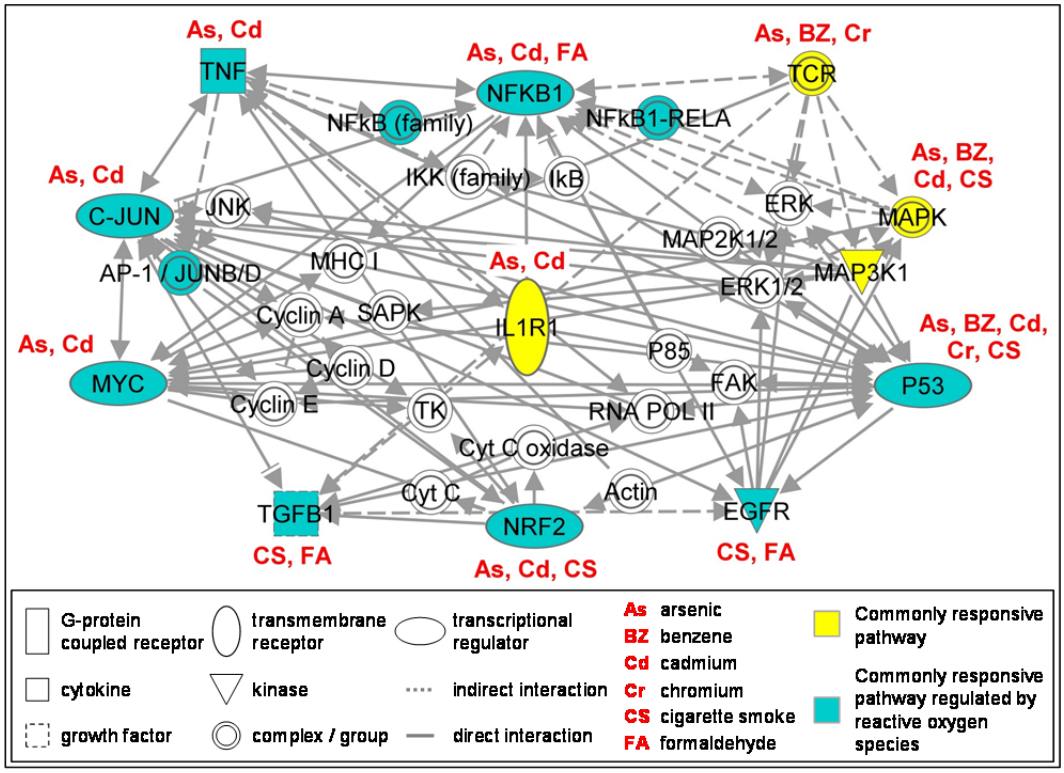

Figure 1. The Environmental Toxicant Signalisome, a network of common responders to various environmental exposures in mammals. Colored proteins represent pathways modified by at least two of the reviewed environmental toxicants. Proteins in white represent signaling associated with the altered pathways.

In order to assess molecular mediators of the Environmental Toxicant Signalisome, an enrichment analysis of upstream regulators was performed, as enabled through the Ingenuity Knowledge Database (Ingenuity Systems®). Reactive oxygen species (ROS) was identified as the most significant (p-value < 6.6×10^{-18}) molecule / molecule group predicted to regulate the environmental exposure-induced pathway alterations. This finding suggests that the Environmental Toxicant Signalisome may be set in motion upon exposure to various toxicants as a result of toxicant-induced ROS production.

The finding that signaling responses to environmental exposures are, at least in part, mediated by ROS production coincides with findings from the reviewed studies. For example, Kawata et al., concluded that genomic responses to six heavy metals coincided with responses induced by DMNQ, an established ROS generating agent (Kawata et al., 2007). In addition, many of the functions associated with systems-level responses to environmental exposures were related to ROS production, including oxidative stress and stress response, which were related to cigarette smoke (Table 3), formaldehyde (Table 5), arsenic (Table 7), cadmium (Table 9), and chromium (Table 11). Further research will be needed to validate the Environmental Toxicant Signalisome as a common responder to other contaminants in mammals.

Discussion and Conclusion

Systems biology can be used to predict chemical toxicity, predict responses to chemicals, identify novel protein interactions, and identify mechanisms underlying exposure-induced disease. The complex relationships between environmental exposures, systems-level responses, and disease continue to be elucidated. As reviewed in this chapter, many functional responses and cellular pathways have been postulated as linking exposures to disease.

Some of the earliest research on this topic was performed using yeast models (Gasch et al., 2000; Jelinsky et al., 1999; Jelinsky et al., 2000). These early works paved the way for systems biology research and set the stage for subsequent studies. In an effort to perform a comprehensive review on the effects of environmental exposures at the systems biology level, we prioritized studies using human and mammalian systems. We urge the reader to examine existing summaries and reviews on systems biology in the context of environmental contaminants applied to other organisms and model systems, such as *S. cerevisiae* (Fry et al., 2005).

As detailed in the present review, transcriptomic-based studies have revealed a depth of knowledge regarding the impact of environmental pollutant exposure on cellular signaling and health. Despite this increased understanding at the systems-level, it is important to recognize that there are limitations in the sole assessment of transcriptional profiles. Transcripts (e.g. mRNAs) must first be translated into proteins, the end product of the gene expression cascade. Proteins are the molecules that ultimately drive cell signaling and function. The evaluation of protein levels should also take into account post-transcriptional regulators and modifications that are not accounted for when assessing transcript levels (Cox et al., 2011).

In order to evaluate the levels of all, or the majority, of proteins within a sample, a proteomics-based approach must be employed. However, this approach has had limited applications thus far in the evaluation of environmental pollutants because of technological difficulties. Some environmental exposure assessment studies have assessed the levels of thousands of proteins, but no studies have been performed assessing the genome-wide protein response to exposure. It was only recently in the yeast model organism that a complete proteome was successfully identified and quantified (de Godoy et al., 2008). In the human, more than 10,000 proteins have been identified to date, but at least 2- to 3-fold more proteins are estimated to exist (Cox et al., 2011). As technologies continue to develop in this area of research, it is likely that future environmental studies will integrate proteomics in order to comprehensively understand the effects of environmental pollutants on cells at the systems biology level.

It is important for research to focus on potential transcriptional regulators of the systems-level responses (e.g. the transcripts). Control of these regulators would have direct application for clinical and therapeutic purposes. There are many ways that the cell can regulate gene expression, through transcription factors as well as epigenetic modifications. Epigenetic modifications include DNA methylation, covalent post-translational histone modifications, and microRNAs (miRNAs) (Bailey et al., 2012). These epigenetic components play critical roles in the regulation of gene expression, acting at either the transcriptional level (DNA methylation and histone modifications) or the post-transcriptional level (miRNAs). There is

increasing evidence that these epigenetic modifications greatly impact human health (Bailey et al., 2012).

Some of the pathway responses to environmental exposures assessed at the transcriptomic-level have been related to DNA methylation alterations. For example, the Waalkes laboratory in 2004 assessed gene expression and DNA methylation levels in the livers of male mice chronically exposed to arsenic in drinking water (Chen et al., 2004). Arsenic was found to alter the expression levels of genes related to apoptosis, cytokines, cell cycle, and steroids. Assessing the amount of global DNA methylation through 5-methylcytosine content revealed that arsenic induced hepatic global DNA hypomethylation (i.e. decreased methylation). Methylation-specific PCR was also used to assess the methylation status within the *ER-α* gene promoter region, where arsenic was found to reduce methylation levels. Findings from this study showed that arsenic in drinking water can cause aberrant gene expression, global DNA hypomethylation, and gene-specific hypomethylation of the *ER-α* promoter region, which can potentially influence arsenic hepatocarcinogenesis (Chen et al., 2004).

Genome-wide gene-specific epigenetic modifications have also been related as systems-level responses to environmental toxicants. For instance, we assessed the DNA methylomes in leukocytes collected from a human cohort in Mexico showing varying signs of arsenicosis (Smeester et al., 2011). A state-of-the-art technique involving the methylated CpG island recovery-chip assay was used to assess DNA methylation levels within CpG islands for over 14,000 genes. A large interactome of hypermethylated genes associated with arsenicosis was identified, containing proteins related to cancer, heart disease, and diabetes. Within this interactome was an arsenic-induced tumor suppressorome, comprising 17 tumor suppressors known to be silenced in human cancer (Smeester et al., 2011). Interestingly, these arsenic-induced epigenetic alterations were later related to inter-individual differences in arsenic metabolism (Bailey et al., 2013). Some of the systems-level responses to toxicants have also been related to miRNA expression alterations. For instance, we established that formaldehyde exposure greatly impacts miRNA expression profiles both *in vitro* (Rager et al., 2011) and *in vivo* (Rager et al., 2013) in direct target tissues of exposure. Transcriptional targets of formaldehyde-modulated miRNAs were also predicted and assessed at the systems-level, where targets were enriched for signaling related to apoptosis, cancer, and inflammation (Rager et al., 2011; Rager et al., 2013). These studies highlight novel mechanisms through which toxicants may cause disease.

While great progress has been made, there are still gaps in the field. For example, many of the reviewed studies evaluated systems-level responses throughout multiple cell types. With cell type separation, the cellular responses that are distinct from toxicant exposure-induced cell shifts can be identified. Additionally, more subtle changes in signaling may not have been detected in mixed cell populations (Waters et al., 2004). Also, few studies have integrated transcriptomic and proteomic analysis from common samples. Such studies would demonstrate which of the transcripts are truly modulated at the protein level. To our knowledge, no phospho-proteomics studies have been carried out to date related to the reviewed environmental toxicants. Interestingly, a phospho-proteomic study has been performed evaluating responses to ROS (Mouzannar et al., 2011), the critical mediator that we identified as associated with the Environmental Toxicant Signalisome. This study identified ROS-associated changes in phosphorylation for proteins contained within the Environmental Toxicant Signalisome, including MAPK signaling members (Mouzannar et

al., 2011). These types of assessments would bring us closer to an understanding of functionally active proteins in the cell.

In conclusion, this chapter provides a summary of nearly 100 studies that contribute critical knowledge towards the understanding of environmental toxicants and their influence on cell signaling at a systems biology level. The results from this review suggest that common machinery is in place and modulated when cells are exposed to environmental toxicants (e.g. here identified as the Environmental Toxicant Signalisome). The better our understanding of such responders, the closer we come to detailed mechanisms of disease that can empower targeted therapeutic interventions to prevent environmental exposure-induced health effects.

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