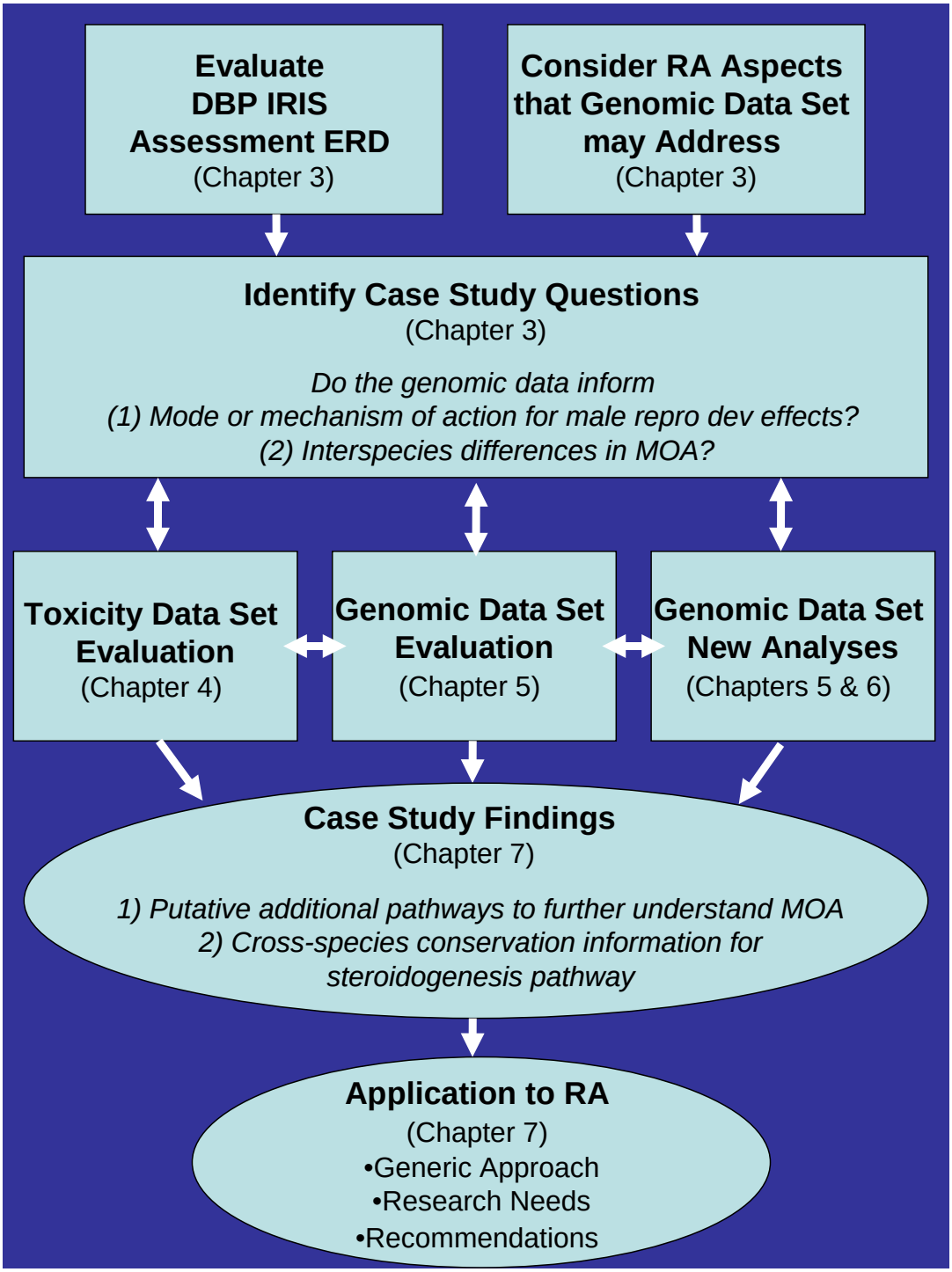

Figure 3-1. DBP case-study approach for evaluating toxicogenomic data for a health assessment. Evaluation steps in the case-study process are shown in rectangles. Findings or products of the case study are shown in ovals. ERD, external review draft. Numbers in parentheses indicate report chapters where the case-study step is described.

Kleymenova et al., 2005a; Barlow et al., 2004; Kim et al., 2004b; Barlow and Foster, 2003; Fisher et al., 2003; Higuchi et al., 2003; Mylchreest et al., 2002, 2000, 1999, 1998; Ema et al., 2000b, 1998, 1997, 1994; Saillenfait et al., 1998).

Figure 3-2 shows the studies that were candidates for the development of the reference dose (RfD) presented in the 2006 external review draft IRIS Tox Review for DBP (U.S. EPA, 2006a). The point of departure (POD) selected for derivation of the RfD for all exposure durations (acute, short-term, subchronic, and chronic) was the no-observed-adverse-effect level (NOAEL) of 30 mg/kg-d for reduced fetal testicular T (Lehmann et al., 2004). In this study, a statistically significant decrease in T concentration in the fetal testis was detected at 50 mg/kg-d. The reduction in fetal testicular T is one of the well-characterized MOAs for DBP that occurs after *in utero* DBP exposure (during the critical window), initiating the cascade of events for a number of malformations in the developing male reproductive tract. Studies using radioimmunoassay of T levels in fetal testes and studies using RT-PCR, microarrays, and/or immunochemical staining found a decrease in the expression of protein and mRNA for several enzymes in the biochemical pathways for cholesterol metabolism, cholesterol transport, and T biosynthesis (also called steroidogenesis more generally) in the fetus (Plummer et al., 2005; Thompson et al., 2004, 2005; Lehmann et al., 2004; Liu et al., 2005; Barlow et al., 2003; Fisher et al., 2003; Shultz et al., 2001). Collectively, these studies document that exposure to DBP disrupts T synthesis in the fetal testis. Thompson et al. (2004) established that following *in utero* exposure to 500 mg/kg-d, the T levels in the testes return to normal after the metabolites of DBP are cleared from the circulation. However, the malformations induced by 500 mg/kg-d exposure persist into adulthood (Barlow et al., 2004; Barlow and Foster, 2003). Thus, although the inhibition of T synthesis can be reversed, the biological effects resulting from the decrease in T during the critical developmental window are irreversible.

3.2. CONSIDERATION OF RISK ASSESSMENT ASPECTS THAT TOXICOGENOMIC DATA MAY ADDRESS

While microarray and RT-PCR data have been used to inform the MOAs of a chemical, the many types of -omic data have the potential to inform TK, dose-response, interspecies and intraspecies differences in TK or TD, and be utilized as biomarkers of exposure or effect (see Figure 3-3).

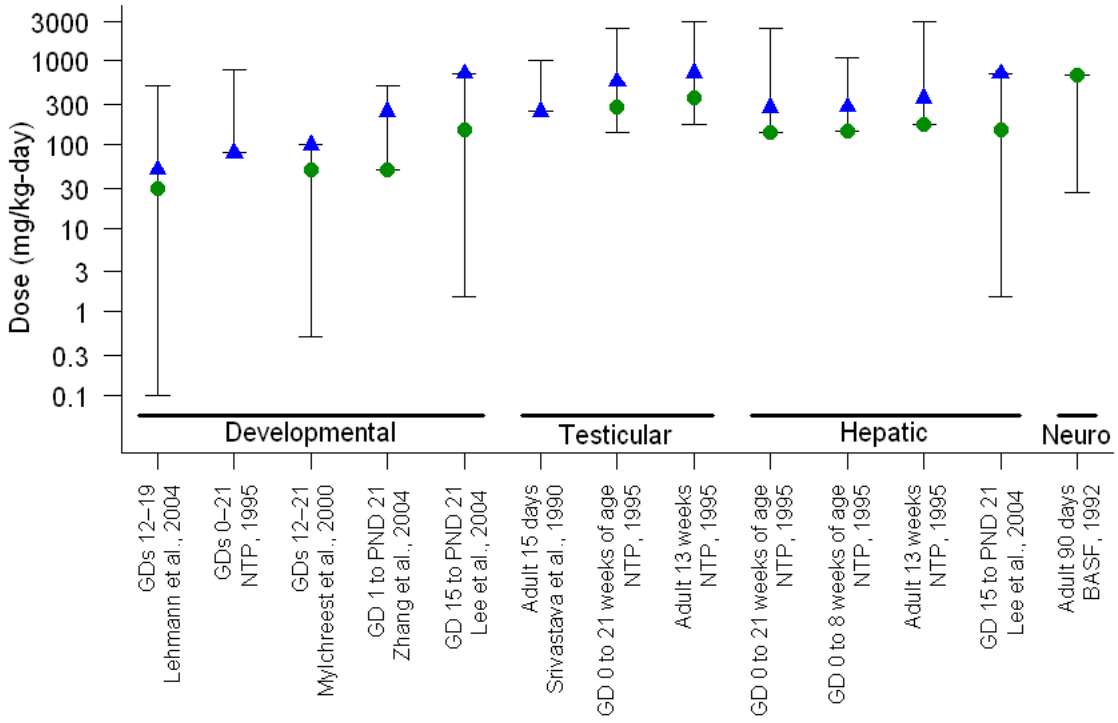

Figure 3-2. Exposure response array for candidate endpoints and PODs for RfD derivation presented in the external review draft IRIS Tox Review for DBP (U.S. EPA, 2006a). The studies are arrayed by toxicological endpoint category. All studies were performed in the rat using the oral route of exposure (although method of oral dosing varied). The study DBP exposure interval and reference are shown on the X axis. The dose (Y axis) is shown in a logarithmic scale. See the articles for additional study details. The vertical line indicates the dose range tested. The lowest and highest doses tested in the study are indicated by the horizontal lines. The green circle indicates the study NOAEL (no-observed-adverse-effect level) and the blue triangle indicates the study LOAEL (lowest-observed-adverse-effect level). Neuro, neurological; GD, gestation day; PND, postnatal day.

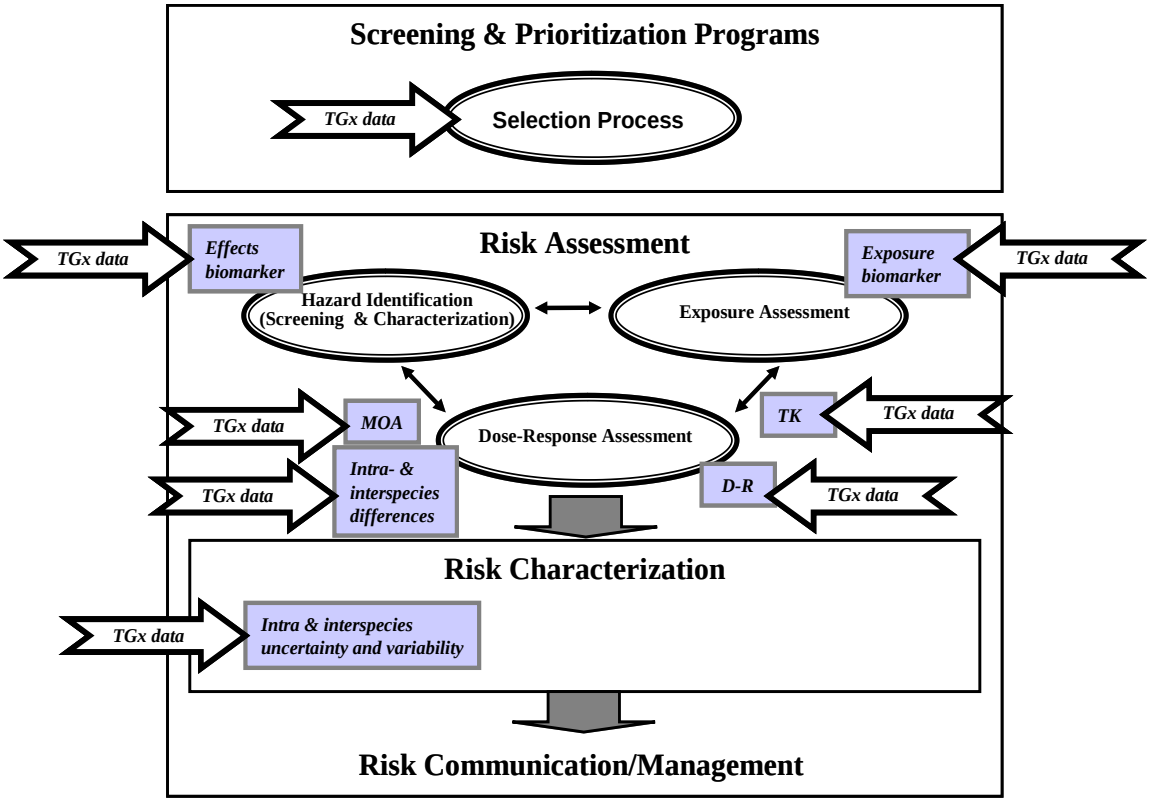

Figure 3-3. **Potential uses of toxicogenomic data in chemical screening and risk assessment.** -Omic data from appropriately designed studies have the ability to inform multiple types of information and in turn, steps in screening and prioritization, and risk assessment. Arrows with TGx data indicate the types of information these data can provide. Shaded boxes indicate some of the types of information that are useful in risk assessment. TGx, toxicogenomic.

However, in this case study, chemical screening and exposure assessment were not considered. Instead, we considered the many types of information useful to hazard characterization, dose-response, and risk characterization. Toxicogenomic data have been successful in providing information about the molecular events altered in the mechanisms of action, and, at times, information about the TD or TK key events of a MOA (see Figure 3-4; see Chapter 2). Further, data from appropriately designed toxicogenomic studies could be used to inform intra- and interspecies differences in molecular responses.

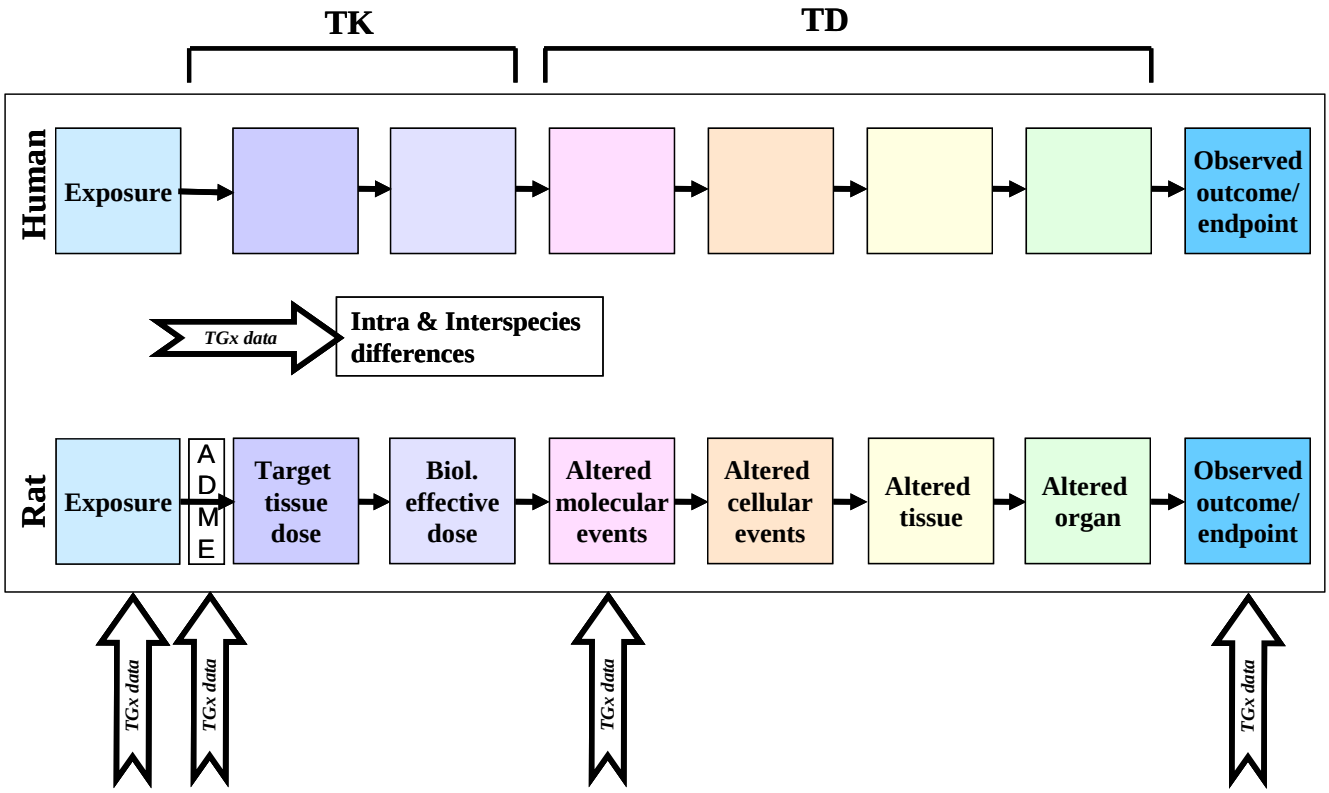

Figure 3-4. **Potential uses of toxicogenomic data in understanding mechanisms of action.** The process from exposure to outcome encompasses all of the steps of a mechanism of action, including both TK and TD steps. Available TGx data, such as microarray data and other gene expression data, can provide information about altered molecular events, at the gene expression level. In turn, appropriate TGx data can be used to inform intra- and interspecies differences in molecular responses. Appropriate TGx data could also inform internal dose and intra- and interspecies differences in internal dose. ADME, absorption, distribution, metabolism, and excretion.

3.2.1. Informing TK

Characterizing the absorption, distribution, metabolism, and excretion (ADME) of environmental toxicants is important for both the understanding and application of MOA information in predicting toxicity. Differences in TK across species, individuals, and exposure patterns (routes, level, duration, frequency) can lead to different biological effects for the same total exposure to a chemical. It is well-established that a quantitative understanding of chemical TK (e.g., using PBPK models) can be useful in analyzing dose-response data and extrapolating across species, individuals, and exposure patterns (U.S. EPA, 2006e). The principles of these uses for TK are the same regardless of the types of response data utilized (i.e., *in vivo* toxicity endpoints [e.g., pup weight] or molecular precursor events [e.g., toxicogenomic changes]), and will not be reviewed here. However, the inverse question of how toxicogenomic data can inform TK has not been fully explored. Here we consider whether toxicogenomic data could be useful for understanding four aspects of a chemical's TK: (1) identification of potential metabolic and clearance pathways; (2) selection of an appropriate dose metric; (3) intra- and interspecies differences in metabolism; and (4) TK/TD linkages and feedback. Each of these applications is discussed below. Finally, the available toxicogenomic data for DBP are evaluated for use in informing TK.

3.2.1.1. ***Identification of Potential Metabolic and Clearance Pathways***

While TK studies, themselves, are designed to help understand the pathways for metabolism and clearance of xenobiotics, toxicogenomic data may provide important complementary information as to what enzymes and tissues may be involved in metabolism. For example, many xenobiotics induce the expression of the Phase I and II enzymes that are responsible for their clearance. Thus, toxicogenomic data showing changes in the expression of genes, such as cytochrome P450s in a particular tissue, may implicate their involvement in metabolizing the compound. While such toxicogenomic data may confirm the major sources of metabolism or clearance, they may also identify minor TK pathways relevant for inducing toxicity. However, data indicating gene expression changes alone are insufficient to conclude that there is a corresponding increase in a protein levels or activity, or are relevant to the ADME of the chemical of interest. Ultimately, toxicogenomic data may be most useful for generating

hypotheses about metabolism and clearance pathways that can be tested with additional TK studies.

3.2.1.2. *Selection of Appropriate Dose Metrics*

Due to inherent differences in TK across species, individuals, and exposure patterns, dose-response relationships are best established based on an internal measure of a biologically effective dose as opposed to an external or applied dose. However, an understanding of TK alone may provide multiple options for the internal "dose metric," such as blood or tissue concentrations of the parent or metabolites, or rates of formation of reactive compounds. Thus, a key question in utilizing TK data for dose-response analyses and extrapolation is dose-metric selection, which depends on the determination of the active chemical species and the MOAs of toxicity. There often may be more than one biologically plausible choice of dose metric, which contributes to the uncertainty in the dose-response analysis. The potential utility of toxicogenomic data is that gene expression data may demonstrate earlier biological effects, and, thus, are closer both spatially and temporally to the interaction between the active chemical species and endogenous cellular molecules than traditional toxicological outcomes (see Figure 3-4). Thus, toxicogenomic data can, in principle, provide biological support for the choice dose metric. Different predictions for internal dose can be statistically analyzed along with toxicogenomic changes that inform TD to determine the dose metric that is best correlated.

3.2.1.3. *Intra- and Interspecies Differences in Metabolism*

Data from polymorphisms is one type of genomic data that can be extremely useful to informing intraspecies differences. Across species, data on differential expression of different isozyme genes may be indicative of differences in overall metabolizing capacity and affinity. In addition, toxicogenomic data may be informative about whether the tissue distribution of metabolizing enzymes may be different across species. Within species, interindividual variability in metabolizing capacity and/or affinity due to differences in enzyme expression or genetic polymorphism can greatly influence the overall TK of a chemical. For example, genetic polymorphisms in aldehyde dehydrogenase-2 (ALDH2) can result in an increase in blood acetaldehyde levels following alcohol consumption, thereby leading to overt health effects (Ginsberg et al., 2002). Similarly, data on CNPs can provide information (Buckley et al., 2005)

that directly informs TK. For example, some individuals possess different copy numbers of CYP2D6 that influence their response to pharmaceuticals (Bodin et al., 2005). When the impacts of gene expression levels and polymorphisms on enzyme levels and function have been established (i.e., preferably confirmed by measurement of enzyme level), this information can either be used to characterize the difference in a predicted dose metric for a subpopulation relative to the most common alleles, or it can be used in probabilistic (e.g., Monte Carlo) analyses to characterize the impact on population variability.

3.2.1.4. *TK/TD Linkages and Feedback*

Ultimately, toxicogenomic data may be useful for linking together TK and TD models into more comprehensive biologically based dose-response (BBDR) models (Daston, 2007). With an appropriate dose metric, one can link the TK predictions for a chemical (e.g., tissue concentration of a metabolite) with toxicogenomic changes (e.g., change in mRNA level) that, in turn, are linked through a TD model to alterations in cellular constituents and, ultimately, frank effects. Furthermore, toxicogenomic data may be useful in providing the link by which the TD feedback of gene and protein expression changes on TK (e.g., enzyme induction) can be modeled.

3.2.1.5. *Research Needs for Toxicogenomic Studies to Inform TK*

Changes in gene expression can be highly labile and vary as a function of dose and time. Thus, identification of appropriate dose metrics involves detection of relevant gene changes as well as the moiety that caused the changes. Therefore, simultaneous data collection of toxicogenomic data and tissue concentrations of the relevant chemical species would be beneficial. In order to inform interspecies extrapolation, it is important to mine toxicogenomic data for potential indicators of species differences in metabolism. For intraspecies variability, it is important to assess the potential impact of polymorphisms in Phase I and II enzymes. Microarray data may also be useful for identifying life stage and gender differences in relative expression of enzymes involved in the TK of the chemical of interest.

3.2.1.6. *DBP Case Study: Do the Available Toxicogenomic Data Inform TK?*

We considered whether the available toxicogenomic data set for DBP informs TK. A greater level of detail is presented for TK in this chapter than for TD because the latter subject is considered in greater detail in the subsequent chapters. This chapter also provides examples of considerations that may be helpful to risk assessors examining whether the available toxicogenomic data can inform TK for their chemical of interest.

The TK of DBP is reviewed in U.S. EPA, 2006a, and is summarized briefly here for context. Following ingestion, DBP is primarily hydrolyzed to monobutylphthalate (MBP) in the gastrointestinal tract and enters systemic circulation through the portal blood. MBP undergoes glucuronidation in the liver, and both free and glucuronidated MBP circulate in serum and are subsequently excreted in urine. While there are a number of TK studies in rats, little human TK data are available, particularly for known exposures to DBP. The available data suggest that free MBP is responsible for the effects on T biosynthesis. In terms of TK pathways, the data set did not lead to the identification of alternative metabolic pathways for DBP.

Toxicogenomic data could inform dose-metric selection in two broad ways: relating the metabolite to the gene expression or using gene expression as the dose metric. In a more traditional approach, changes in the expression for genes of interest could be related to a chemical moiety in a target tissue of relevance (or convenience). For example, Lehmann et al. (2004) provides a dose-response analysis of gene expression following DBP exposure. However, these data are limited for use in extrapolation without TK data (e.g., tissue concentrations of MBP). Ideally, TK data could be collected at various time points following various doses, but this would require a large number of fetuses. In the absence of such empirical data, analyses could be performed using physiologically based TK modeling, but none have yet been attempted. Such an approach might utilize the available published TK studies for DBP and attempt to reconstruct the exposure scenarios in the toxicogenomic studies with the intent to predict the MBP concentration in a target tissue (or blood) at the developmental time points where toxicogenomic samples were obtained.

A second and more complex approach might be to use a toxicogenomic change as a dosimeter (or "biomarker"), which may obviate the need for TK data altogether. For example, the microarray study of Wyde et al. (2005) reports changes in maternal liver *Cyp2b1* and *Cyp3a1*, and estrogen sulfotransferase mRNA levels following DBP exposure. Not only do

these gene expression changes serve as potential biomarkers, but they also suggest that there may be related changes in metabolic biomarkers (i.e., metabonomics) because these enzymes have roles in lipid and hormone synthesis, in addition to xenobiotic metabolism. Although it is not clear whether these changes have a relationship to a toxic endpoint of interest, it may be possible to establish, for instance, that an increase in a specific maternal liver mRNA is correlated with a decrease in a specific mRNA in the fetal testis. Indeed, Wyde et al. (2005) show that maternal liver estrogen sulfotransferase gene expression increases in a dose-dependent manner from 10−500 mg/kg-d, while Lehmann et al. (2004) observed a dose-dependent decrease in *Scarb1*, *Star*, *Cyp11a1*, *Hsd3b*, or *Cyp17a1* mRNA levels in fetal testes from 0.1–500 mg/kg-d.

With respect to interspecies extrapolation and interindividual variability, the lack of adequate human TK data precludes quantitative extrapolation, a situation that cannot be remedied with the available toxicogenomic data (unless, as discussed above, a toxicogenomic-based dosimeter/biomarker was developed). For instance, available blood measurements of MBP in humans were taken from spot samples in the general population where the individual exposure patterns were unknown. Although differences were observed in the ratio of free to conjugated MBP in human serum as compared to the rat, these data are insufficient for quantitative interspecies extrapolation, because in order to replace administered dose as a dose metric, it is necessary to determine the *absolute*, not the *relative*, level of free MBP in serum as a function of exposure. The Wyde et al. (2005) study suggests that DBP-induced enzyme induction occurred. Specifically, this study reported that exposure to 50 and 500 mg/kg-d DBP leads to an increase in rat liver UDP glucuronsyltransferase 2B1 (*Ugt2b1*) mRNA levels. More TK analysis would be required to ascertain whether this induction in rats occurs at levels that are relevant to low-dose exposures. This enzyme induction may occur in humans and such a response may increase interindividual sensitivity to DBP toxicity. With regard to human TK, none of the available toxicogenomic data on DBP were performed in humans and thus, do not provide any information on DBP interindividual TK variability. For example, there are no available data on polymorphisms in glucuronyltransferases responsible for metabolizing MBP. Finally, we considered the potential for TK/TD linkages with the available data and concluded that in order for TK and toxicogenomic data to be integrated for use in quantitative dose-response analysis, more sophisticated BBDR models are needed. Using such an approach,

it may be feasible to relate changes in expression of genes involved in T production to quantify testicular T levels (see Figure 3-5).

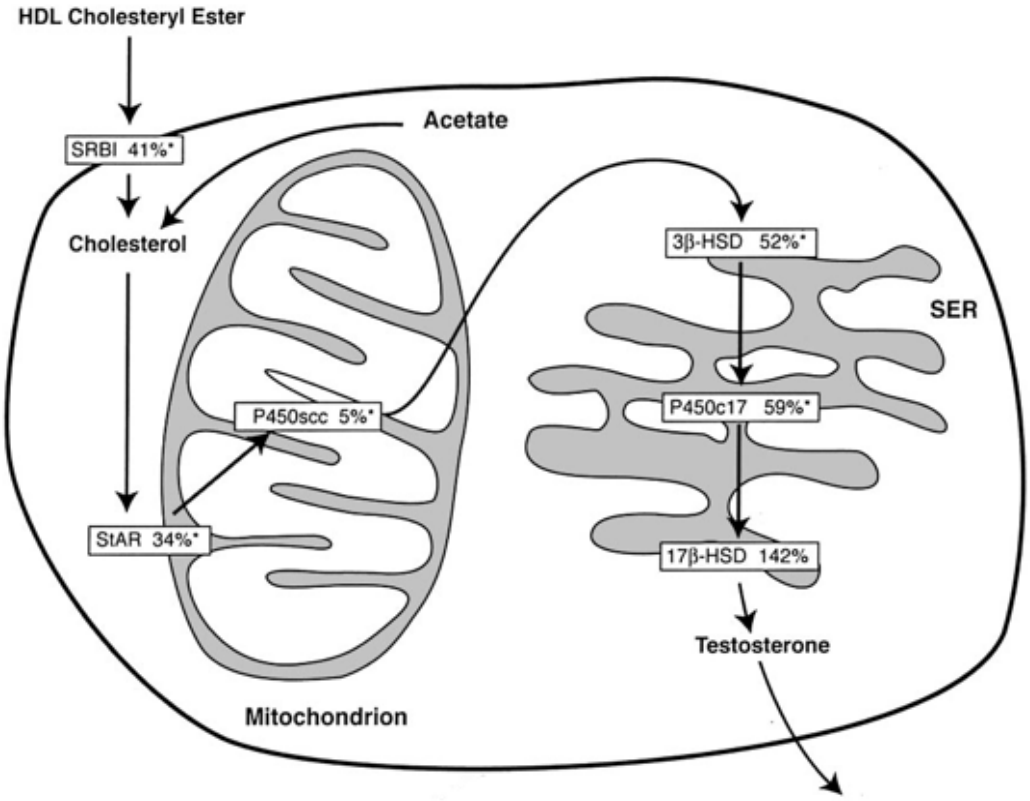

Figure 3-5. **The fetal Leydig cell in the fetal testis.** The boxes represent genes involved in the biosynthesis of T; the percentages (%) represent % control gene expression in fetal testis of dams treated with 500 mg/kg-d DBP.

Source: Adapted from Barlow et al. (2003).

The male reproductive developmental effects of DBP appear to be mediated by MBP, which causes a down-regulation of cholesterol transporters across the cell membrane (SCARB1) and mitochondrial inner membrane (STAR), as well as the down-regulation of two enzymes involved in converting cholesterol to T, CYP11A1, and CYP17A1 (Liu et al., 2005; Lehmann et al., 2004; Barlow et al., 2003; Shultz et al., 2001). Thus, it may be possible to relate DBP and/or MBP levels to reductions in cholesterol transporter (e.g., SCARB1 and STAR) and the levels of steroidogenic enzymes (e.g., CYP11A1 and CYP17A1) at the mRNA, protein, and/or activity

levels. Changes in these parameters may then be modeled to predict changes in testicular T levels, which may subsequently be correlated to developmental toxicity endpoints.

3.2.2. Informing Dose-Response

Toxicogenomic data that informs TK can be useful for informing or improving dose-response analysis because it may improve the dose metric selection among alternative dose metrics. However, use of toxicogenomic data as an endpoint in dose-response analysis has not been extensively explored. For example, BMD analysis of some dose-response studies determined PODs based on the GO categorization of gene expression changes (based on an approach of Yu et al., 2006) as a function of dose (Andersen et al., 2008; Thomas et al., 2007).

3.2.2.1. *DBP Case Study: Do the Toxicogenomic Data Inform Dose-Response?*

Unfortunately, there are currently no available dose-response microarray studies to assess the genome-wide gene expression over a dose range. However, there is one available dose-response gene expression study for DBP. Specifically, Lehmann et al. (2004) conducted a dose-response study evaluating testicular T, RT-PCR and protein expression for a subset of genes thought to underlie the male reproductive developmental outcomes. This study reported a significant reduction in fetal testicular T at 50 mg/kg-d DBP or higher. Western analysis found that steroidogenic acute regulatory protein (STAR) and scavenger receptor class B, member 1 (SCARB1) were significantly decreased at 50 mg/kg-d while cytochrome P450, family 11, subfamily a, polypeptide 1 (CYP11A1) was only reduced at 500 mg/kg-d. Further, RT-PCR analysis findings confirmed that the mRNA of these three genes was statistically significantly reduced at 50 mg/kg-d. The results of this study support the role of steroidogenesis enzymes and cholesterol transport proteins in the decreased testicular T MOA after *in utero* DBP exposure. However, without first establishing the biologically significant level of change in gene expression and the critical subset of genes that constitute a well-established precursor event, it is difficult to use these data in a dose-response assessment (see Chapter 7). It would be helpful to have dose-response microarray or proteomic studies to assess mRNA and protein expression on a genome-wide level.

3.2.3. Informing TD

There are numerous examples where toxicogenomic data have been used to inform the TD steps within mechanisms of action or MOAs for a chemical, and there are a small number of examples where such data have been used corroboratively for risk assessment decisions (see Chapter 2).

3.2.3.1. *General Considerations: TD Portion of Mechanisms of Action and MOAs*

One feature of the approach (see Figure 3-1) is the evaluation of the toxicity and toxicogenomic data sets in conjunction in order to consider the relevance of gene expression changes with respect to specific endpoints of interest identified in the toxicity data set. In this manner, data on affected pathways may generate hypotheses and inform the mechanisms of action for a chemical for specific endpoints. In addition, using this approach could provide connections between affected pathways (identified from the toxicogenomic data set) and endpoints (identified from the toxicity data set), which may, in turn, inform modes or the mechanisms of action, as illustrated by Figure 3-6. Chapter 2 and the glossary describe the distinction between the definitions for mechanisms of action and MOAs.

This approach is best suited to instances where comparable study designs between the toxicity/epidemiology and toxicogenomic data sets are available. For example, toxicogenomic and toxicity studies performed in the same species, using similar doses, similar exposure intervals, and assessing the same organ or tissue would be ideal for utilizing this approach. For the DBP toxicity (see Chapter 4) and toxicogenomic data sets (see Chapter 5), there is some comparability across some of the studies—i.e., some toxicity and toxicogenomic studies were performed at the same doses with similar exposure intervals, in the same species and strain, and assessed some of the same organs (e.g., testis). However, no two studies are the same for all study-design aspects, such as precise timing of exposure and time of assessment.

3.2.3.2. *DBP Case Study: MOAs for Male Reproductive Developmental Effects*

Developmental toxicity studies (reviewed in Chapter 4) and toxicogenomic studies (reviewed in Chapter 5) have contributed to a good understanding of DBP as a chemical that has multiple MOAs. Two well-characterized MOAs: a reduction in fetal testicular T and a reduction in *Insl3* signaling activity, explain a number of the observed male reproductive developmental

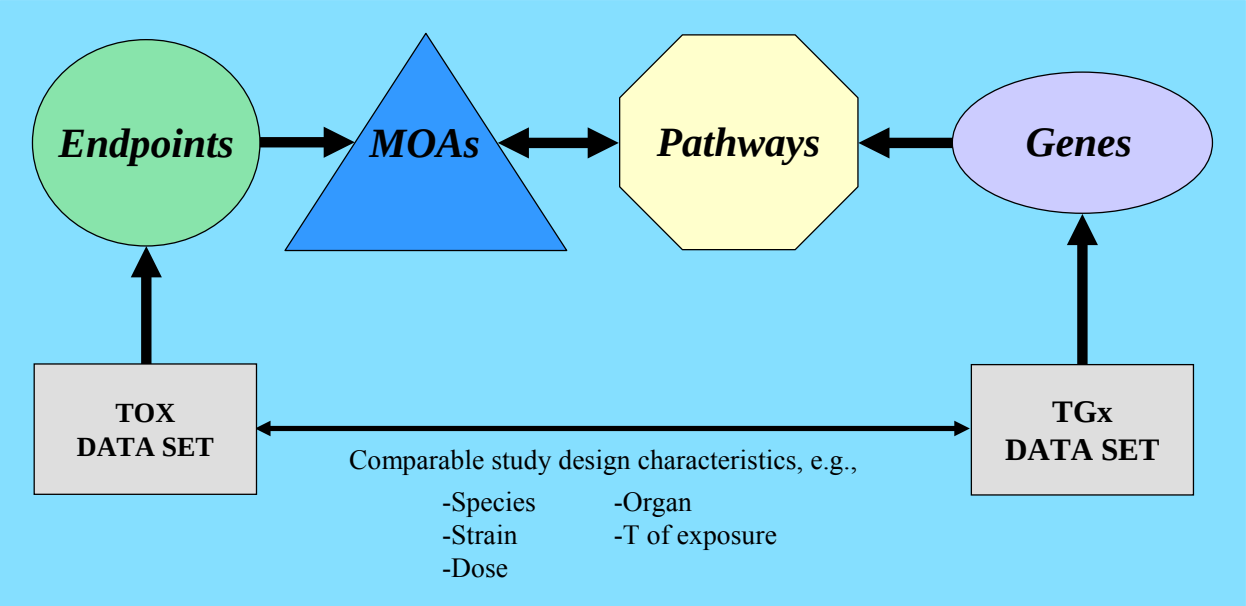

Figure 3-6. **Approach to utilizing toxicity and toxicogenomic data for identifying affected pathways and candidate modes and mechanisms of action.** Toxicogenomic data can be analyzed for differentially expressed genes (DEGs) and, in turn, grouped into affected pathways. Toxicity data can provide information about affected endpoints. Evaluating toxicogenomic and toxicity data together can provide a level of phenotypic anchoring between gene and pathway changes, and *in vivo* outcomes. The identification of affected pathways can inform mechanisms of action, including MOAs, for a chemical. Such an approach requires similar study parameters (e.g., dose, species, duration of exposure) for the toxicity and toxicogenomic studies. TGx, toxicogenomic.

abnormalities. Some other observed abnormalities are not explained by these two MOAs, suggesting that there are additional MOAs for DBP. Acknowledging that there are additional data not presented in Figure 3-7, this figure attempts to show where there is agreement in the scientific community, based on reproducibility of microarray and RT-PCR studies, about affected pathways and the well-characterized MOAs for DBP. There are some endpoints and pathways that need further characterization and, as a result, we were interested in determining whether the toxicogenomic data could be used to associate the DBP MOAs and endpoints, and/or form hypotheses about additional MOAs for DBP.

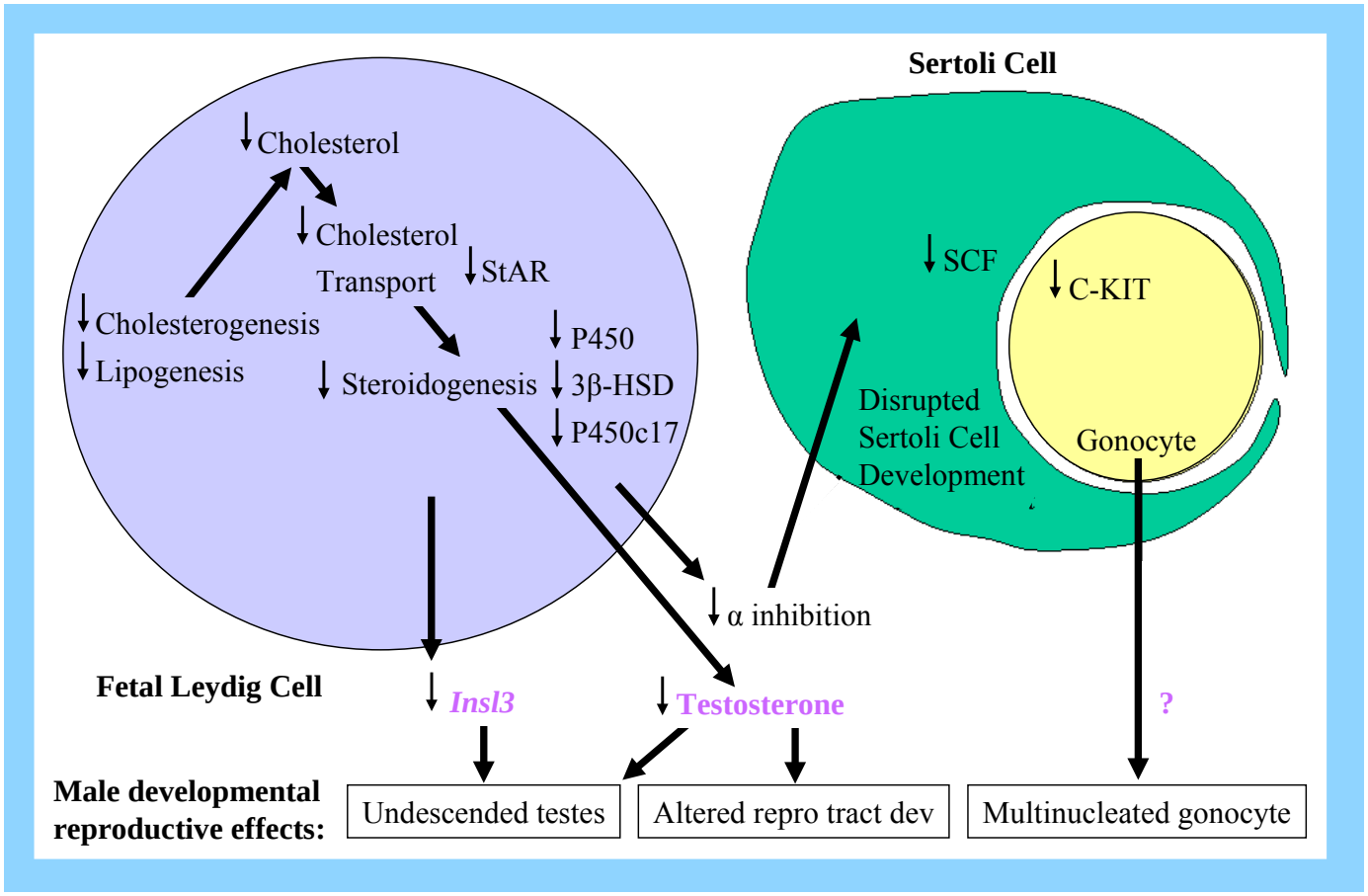

Figure 3-7. **The proposed DBP mechanism of action for the male reproductive developmental effects.** The mechanism of action is defined as all of the steps between chemical exposure at the target tissue to expression of the outcome. The steps shown are based on male reproductive developmental toxicity and toxicogenomic studies. Some of the affected pathways and individual genes whose expression was significantly affected after DBP exposure in multiple studies are included. By contrast, MOAs are shown in purple letters including two well-characterized MOAs and one example of an unidentified MOA.

Source: Figure adapted from Liu et al. (2005), Thompson et al. (2004), Wilson et al. (2004), Barlow et al. (2003), and Shultz et al. (2001).

3.3. IDENTIFYING AND SELECTING QUESTIONS TO FOCUS THE DBP CASE STUDY

In reviewing the external review draft IRIS Tox Review for DBP, data gaps in the assessment were noted. Then the DBP toxicogenomic data set was evaluated for these data could potentially address any of the gaps (see Figure 3-1). The identified data gaps led to formulation of questions of interest whose answers may be able to contribute valuable information to a risk assessment. The following questions were identified:

Can the DBP toxicogenomic data set inform the

- **biologically significant level of reduction in fetal T?** As the external review draft IRIS Tox Review for DBP used a reduction in fetal testicular T as the critical effect, we considered whether the toxicogenomic data set could aid in determining the biologically meaningful level of T reduction for the male reproductive developmental effects.
- **dose-response assessment in risk assessment?** The microarray and RT-PCR studies have identified genes and pathways associated with the reduced fetal testicular T. Thus, there is the potential for evaluating these genes and pathways in a dose-response assessment.
- **modes and mechanisms of action for male reproductive developmental outcomes?** Not all of the male reproductive developmental outcomes after *in utero* DBP exposure are a consequence of reduced fetal testicular T or reduced *Insl3* expression. Therefore, additional MOAs for these endpoints may be identified from pathway analysis of the microarray data.
- **interspecies (rat-to-human) differences in MOAs that could, in turn, inform the TD part of the UF_H?** There is evidence from toxicogenomic studies that a reduction in gene expression of some of the steroidogenesis genes underlies the reduction in fetal testicular T observed after *in utero* DBP exposure. Unfortunately, there are no genomic studies in appropriate human *in vitro* cell systems to make comparisons to *in vivo* rat MOA findings. Using available DNA sequence data and other methods, we would like to assess the rat-to-human conservation of the steroidogenesis pathway genes.

The existing genomic data for DBP had the potential to inform two of the questions: informing modes and mechanisms of action and interspecies differences for the reduced T MOA (see above). It was highly likely that the DBP toxicogenomic data set could aid in hypothesis generation of DBP modes or mechanisms of action. Using genomic data, such as DNA sequence data, it may be possible, but less likely, to inform cross-species differences in TD for the reduced T MOA. Although the other two questions (see list above) were of great interest, the available genomic data were not considered appropriate to address them.

The remaining steps of the DBP case study are presented in the subsequent chapters. The evaluations of the toxicity data set for the male reproductive developmental effects after developmental exposure to DBP (see Chapter 4) and the toxicogenomic data set including new analyses of one microarray study (see Chapter 5). Exploration of pathway analysis methods development for applying microarray data to risk assessment and the use of available methods to evaluate rat-to-human differences for the reduced T MOA are presented in Chapter 6. Chapter 4 follows with an in-depth evaluation of the DBP toxicity data set.