



**Genetic Properties Influencing the Evolvability of Gene Expression**

Christian R. Landry, *et al.*  
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tions cannot be rejected by the approximately unbiased (AU) multiscale bootstrap test (20) at the 5% level (fig. S2). This uncertainty may relate to the fact that *Buddenbrockia* genes have undergone rapid sequence evolution, which can either cause artifactual groupings or reduce the support for the correct grouping (21, 22). This is not expected to be the cause of the grouping between *Buddenbrockia* and Medusozoa, because the branches of both the Hydrozoa and Scyphozoa species are short and should not act as a long-branch attractor. When trees were inferred by parsimony, a method highly susceptible to long-branch attraction (23), *Buddenbrockia* was grouped with an artifactual clade of long-branch platyhelminths and nematodes, not Medusozoa (fig. S3). To circumvent the long-branch attraction effect (24, 25), we reanalyzed the data under the CAT model, which explicitly handles the heterogeneity of the substitution process across positions (26). The CAT tree (fig. S4) was identical to the Bayesian tree except for the relative placement of some nonmetazoan branches. It is noteworthy that less phylogenetic resolution was observed within the *Buddenbrockia* + Medusozoa clade, as these results suggest that *Buddenbrockia* is either an outgroup to Scyphozoa plus Hydrozoa (83% CAT) or sister to Hydrozoa (17% CAT). On the basis of these data, we conclude that the *Buddenbrockia* worm is a cnidarian. This conclusion can be extrapolated to all Myxozoa, because previous work has established that *Buddenbrockia* is a member of this clade (7, 9). Therefore, the taxon Myxozoa should be placed within the phylum Cnidaria, on the medusozoan lineage.

Our data also show that, not only has anatomical simplification occurred in myxozoan evolution, but so has evolution of a muscular vermiform body. We infer that active, motile worms are not restricted to the bilaterian animals, but can be found among the cnidarians. One interpretation is that the common ancestor of cnidarians and bilaterians had a muscular worm-shaped body plan. However, this does not seem compatible with the ultrastructure of *Buddenbrockia* or the phylogenetic distribution of vermiform animals. Instead, we hypothesize that the muscular, motile worm form evolved independently within cnidarians, by means of a loss of the opening to the gastrovascular cavity and subsequent acquisition of a hydrostatic skeleton. Parallel evolution of the vermiform body may have exploited a conserved developmental system for patterning an ancestral mesodermal layer homologous between Bilateria and Cnidaria. (27)

Ultrastructural studies reveal that the four blocks of well-defined longitudinal muscles in *Buddenbrockia* are radially distributed (Fig. 1) (12). Hence, *Buddenbrockia* is a tetradial worm with two axes of symmetry across a transverse section, not a bilaterally symmetrical worm with one axis of symmetry. Bilateral symmetry was long thought to be associated with the evolution

of directed locomotion, perhaps in an ancestral bilaterian. This view is challenged by the existence of subtle bilateral symmetry in sessile anthozoan cnidarians (28, 29); hence, it has been suggested that bilateral symmetry arose through selection for effective internal circulation not directed locomotion (30). The finding that an active muscular worm evolved within the Cnidaria, yet retained radial symmetry, is consistent with this view, because it further dissociates locomotion from symmetry. *Buddenbrockia* is a worm, but not as we know it.

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#### Supporting Online Material

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Materials and Methods  
Figs. S1 to S4  
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References

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## Genetic Properties Influencing the Evolvability of Gene Expression

Christian R. Landry,<sup>1\*†</sup> Bernardo Lemos,<sup>1\*‡</sup> Scott A. Rifkin,<sup>1‡</sup> W. J. Dickinson,<sup>2</sup> Daniel L. Hartl<sup>1</sup>

Identifying the properties of gene networks that influence their evolution is a fundamental research goal. However, modes of evolution cannot be inferred solely from the distribution of natural variation, because selection interacts with demography and mutation rates to shape polymorphism and divergence. We estimated the effects of naturally occurring mutations on gene expression while minimizing the effect of natural selection. We demonstrate that sensitivity of gene expression to mutations increases with both increasing trans-mutational target size and the presence of a TATA box. Genes with greater sensitivity to mutations are also more sensitive to systematic environmental perturbations and stochastic noise. These results provide a mechanistic basis for gene expression evolvability that can serve as a foundation for realistic models of regulatory evolution.

Regulatory variation underlies much of phenotypic diversity, and gene expression is the first step in making ecologically and evolutionarily relevant phenotypes. Differences among genes both in standing genetic variation and in interspecies divergence in gene expression have been linked to their particular roles in biological networks (1–4) and may reflect a history of selection. However, the influence of specific evolutionary forces cannot

be inferred solely from the distribution of natural variation, because selection interacts with demography and mutation to shape polymorphism and divergence (5). Measuring the effects of spontaneous mutations without the confounding effect of natural selection makes it possible to isolate the contribution of mutation to natural variation and is a fundamental step toward building models for the evolution of gene expression. The relationship between divergence and muta-

tional effects on gene expression has been measured in the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* (6, 7), revealing that stabilizing selection plays a dominant role in limiting the extent of polymorphisms in gene expression in nature (8). We used *Saccharomyces cerevisiae* to investigate how the structural properties of genes and regulatory networks shape the relation between mutations and gene expression and thereby affect the course of evolution.

We performed a mutation-accumulation (MA) experiment (Fig. 1A) in *S. cerevisiae* in order to isolate the contribution of the mutational process to gene expression evolution. With serial transfer of random colonies, we accumulated spontaneous mutations by maintaining parallel lines with

<sup>1</sup>Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

<sup>2</sup>Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA.

\*These authors contributed equally to this work. To whom correspondence should be addressed. E-mail: clandry@post.harvard.edu (C.R.L.); blemos@oeb.harvard.edu (B.L.)

†Present address: Département de Biochimie, Université de Montréal, 2900 Boulevard Édouard-Montpetit, Montréal, QC H3T 1J4, Canada.

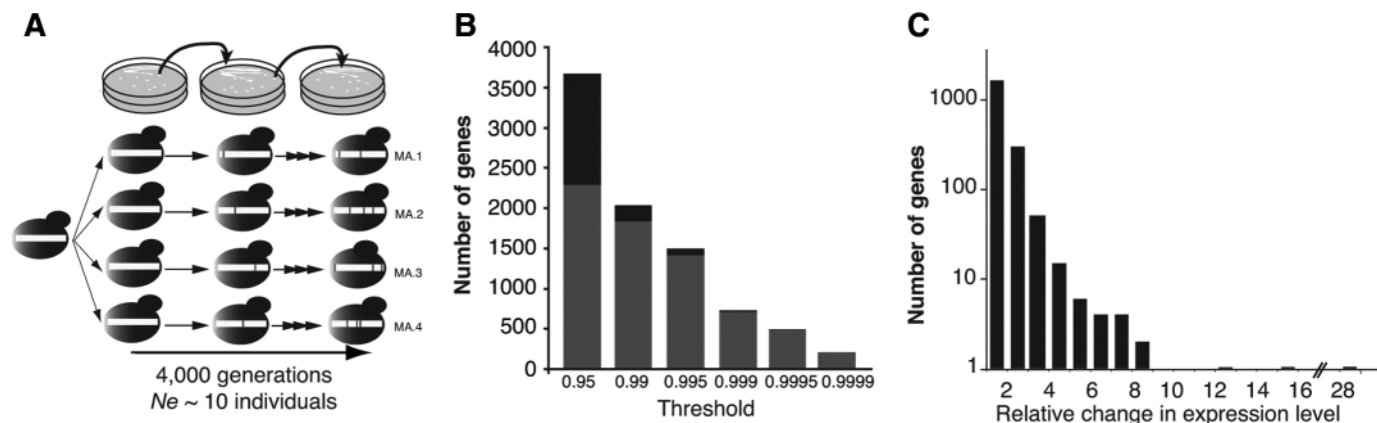
‡Present address: Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

effective population sizes of  $\sim 10$  individuals. The lines diverged from an isogenic common ancestor for 4000 generations. At this population size, the fate of most nonlethal mutations is largely governed by random genetic drift (9), and the divergence observed among the lines allows us to estimate the rate at which gene expression would evolve in the near absence of selection. Lethal mutations would be eliminated through our experimental protocol, but they are unlikely to contribute to standing genetic variation produced by mutations in natural populations. We randomly selected four MA lines, measured their gene expression levels with DNA microarrays (10), and estimated rates of gene expression evolution.

The rate of phenotypic evolution due to mutation alone can be measured by the mutational variance ( $V_m$ ), which is defined as the increase in the variance of a trait introduced by mutations each generation. It can be calculated from the variance of traits among MA lines. For haploid asexual organisms,  $V_m = 2\sigma_b^2/t$ , where  $\sigma_b^2$  is the between-line variance and  $t$  is the number of generations (5). We estimated the  $V_m$  of gene expression for genes that showed significant statistical differences (Bayesian posterior probability  $> 0.99$ ) in expression among any pair of the four MA lines by using log-

transformed relative expression levels (Fig. 1B). This resulted in 2031 genes differentially expressed across strains, with 85 showing differences higher than threefold (Fig. 1C). We found that the median  $V_m$  in gene expression in yeast is  $4.7 \times 10^{-5}$  (fig. S1), which is comparable to that previously estimated in fruit flies [ $\sim 2 \times 10^{-5}$  (6)] and about two orders of magnitude below those typically observed for morphological phenotypes (11). Hence, there are common characteristics that determine the mutational variation in gene expression in spite of large differences between these organisms. Furthermore, our estimates of  $V_m$  correlate positively with genetic variation in gene expression among natural isolates of *S. cerevisiae* (12) ( $\rho = 0.25$ ,  $P < 2.2 \times 10^{-16}$ ,  $n = 1888$ ). Therefore, variation in levels of expression among genes and regulatory pathways in natural populations are shaped in part by variation in the transcriptional sensitivity to mutations. Also, we found that genes with high  $V_m$  tend to be underrepresented in biological processes such as cell growth and maintenance, metabolic process, cell cycle, and transcription (fig. S2).

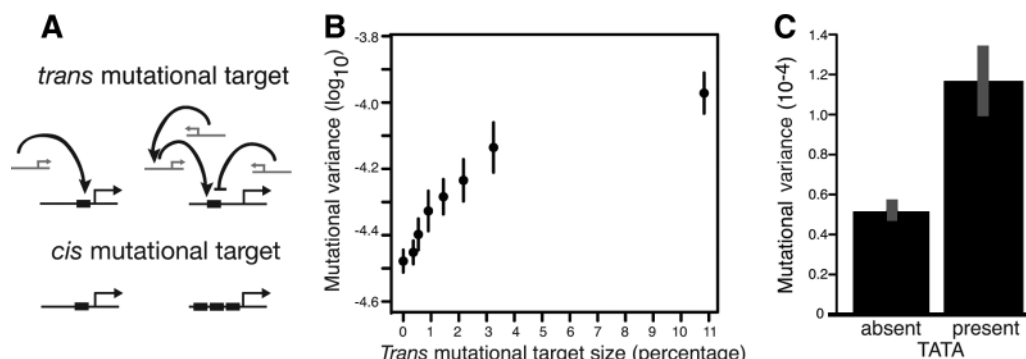
Three main factors influence the probability that a mutation affects the expression level of a gene: (i) the number of other genes that influence the expression of the focal gene (trans-mutational



**Fig. 1.** (A) MA experimental design. (B) Number of genes differentially expressed among the four MA lines as a function of the Bayesian posterior probability of differential expression. Black bars indicate the es-

timated fraction of genes expected by chance. (C) Relative-fold change in expression level for genes with significant differences among the four MA lines.

**Fig. 2.** (A) Schematic of trans- and cis-mutational target sizes. On the left of each image are cases of smaller mutation target sizes, and on the right are larger mutation target sizes. The trans-mutational target size does not solely include transcription factors but all genes acting upstream of the focal gene. (B) Positive relationship between trans-mutational target size and  $V_m$ . The averages of 10 bins are plotted, with error bars denoting two standard errors. (C) Mean  $V_m$  of genes with and without a TATA box in their promoters. Error bars denote two standard errors.



target size), (ii) the number of regulatory elements controlling the expression of the gene (cis-mutational target size), and (iii) the distribution of effects of mutations on expression. We examined whether features of these first two components could affect the sensitivity of expression levels to mutation (Fig. 2A).

The trans-mutational target size of a gene is composed of the number of genes in the genome that affect the expression level of the focal gene, weighted by their influence and their own mutational parameters (Fig. 2A). We used expression profiling of 297 gene knockouts (13, 14) to estimate the trans-mutational target size of a gene as the fraction of deletions of other genes in the genome that affect its expression level. We found that  $V_m$  correlates strongly with the trans-mutational target size ( $\rho = 0.33$ ,  $P < 2 \times 10^{-16}$ ,  $n = 1951$ ) (Fig. 2B and fig. S3). Hence, larger trans-mutational target sizes may indeed result in higher sensitivities of gene expression to mutations.

The cis-mutational target size of a gene scales with the number and sizes of transcription factor binding sites, either directly through the number of nucleotides in the sites or indirectly through the number and variety of regulatory molecules binding to these sites. We mapped transcription factor binding sites to yeast promoters and determined the number of binding sites per promoter (10, 15). Genes that changed significantly in expression among the MA lines had a larger number of binding sites than those that did not change significantly (2.9 versus 2.4, Wilcoxon rank test,  $P = 1 \times 10^{-5}$ ), and the  $V_m$  globally increased with the number of binding sites ( $\rho = 0.14$ ,  $P = 0.0007$ ,  $n = 608$ ). Genes with a large number of transcription factor binding sites are more sensitive to spontaneous mutations affecting the level of gene expression.

Eukaryotic genes differ in the composition of their cis-regulatory targets. About one-fifth of yeast genes contain a TATA box (16), which modifies several aspects of their transcriptional regulation (17, 18). TATA-containing genes are more likely to be subtelomeric, highly regulated

by nucleosomes and chromatin regulators (16), and associated with elevated rates of gene-expression divergence among species (4) and adaptation during experimental evolution (16). This divergence may be the result of diversifying selection (4, 16), but it could also reflect a bias in the sensitivity of TATA-containing genes to spontaneous mutations.

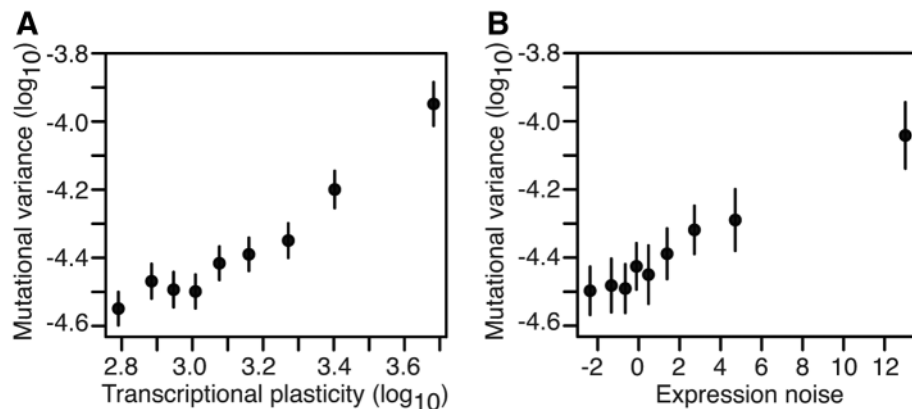
We found that genes with a TATA box were significantly more likely to change in expression among the MA lines (49% versus 32%; Fisher's exact test,  $P = 2.5 \times 10^{-16}$ ) (table S1) and had a mean  $V_m$  that was twice as high as that of genes lacking a TATA box ( $V_m$  of  $1.17 \times 10^{-4}$  versus  $0.52 \times 10^{-4}$ ; Wilcoxon rank test,  $P < 2 \times 10^{-16}$ ) (Fig. 2C). Although stress-response genes are particularly enriched for TATA boxes (4, 18), eliminating stress-response genes from the analysis did not change the result ( $V_m$  of  $1.0 \times 10^{-4}$  versus  $0.51 \times 10^{-4}$ ; Wilcoxon rank test,  $P < 2.2 \times 10^{-16}$ ). Hence, genes with a TATA box are more sensitive to genetic perturbations, and their overrepresentation among genes responding rapidly to artificial selection (16) and genes that show increased divergence among species (4) can be partly explained by their higher regulatory evolvability. Indeed, the larger trans-mutational target sizes of TATA-containing genes (0.02 versus 0.007; Wilcoxon rank test,  $P = 3 \times 10^{-16}$ ) suggest a mechanism by which this may be achieved.

Because TATA box-containing genes have large cis- and trans-mutational target sizes relative to TATA-less genes, we used a series of generalized linear models to simultaneously assess the effects of the trans- and cis-mutational target sizes and the presence of a TATA box on the sensitivity of expression levels to mutations. First, we found that the larger number of binding sites in the promoters of TATA-containing genes [(4); in our data set, 3.3 versus 2.2; Wilcoxon rank test,  $P = 7 \times 10^{-11}$ ] could fully account for the previous correlation between cis-mutational target size and  $V_m$ . When other factors are considered simultaneously, the number of tran-

scription factor binding sites has no effect on the  $V_m$  of the gene (0.5% of the variance explained,  $P = 0.11$ ). Second, we found that the larger trans-mutational target size of TATA-containing genes cannot fully account for the relationship between trans-mutational target size and  $V_m$ . Instead, trans-mutational target size and  $V_m$  are associated even when the effects of the TATA box are first removed (tables S2 and S3). Furthermore, we find a significant correlation between the  $V_m$  and the trans-mutational target size even after excluding TATA-containing genes ( $\rho = 0.2$ ,  $P < 0.00001$ ,  $n = 811$ ), thus lending unambiguous support to the conclusion that effects of trans-mutational target size are independent of the TATA box.

A fundamental feature of organisms is their capability to cope with genetic and environmental perturbations (19). Whereas genetic and environmental canalization have often been conceptualized (20) and modeled (21) as distinct phenomena, mechanisms that produce canalization may act simultaneously to modulate the effects of both kinds of perturbations (22). Hence, phenotypes that are buffered against the effects of environmental perturbations might also be buffered against the effects of mutations. With public data on the amount of variation in gene expression over different environmental conditions (4), we found that  $V_m$  in the expression of a gene and its transcriptional plasticity to macro-environmental perturbations are positively correlated (Fig. 3A) ( $\rho = 0.37$ ,  $P = 2 \times 10^{-16}$ ,  $n = 1735$ ). Furthermore, we found that protein expression noise, a measure of the sensitivity of gene expression to microenvironmental variation such as fluctuations in the amount of upstream cellular components (23–25), and  $V_m$  are also positively correlated (Fig. 3B) ( $\rho = 0.27$ ,  $P = 1 \times 10^{-14}$ ,  $n = 776$ ). These relationships are not confounded by the effects of gene expression level, because neither mRNA nor protein abundances correlate with  $V_m$  (fig. S4, A and B). Hence, the effects of mutational and both environmental perturbations and stochastic noise are related such that mechanisms that evolve to promote or buffer transcriptional responses to one source of variation may also affect the others. Lastly, the strength of the relationship between environmental and genetic perturbations vary across sets of genes (table S4 and SOM), indicating that the relative contributions of these sources of perturbation toward the evolution of canalization may differ substantially from one gene or metabolic network to the next.

We show that not all genes are equally sensitive to the effects of random spontaneous mutations and identify structural properties (presence of a TATA box and trans-mutational target sizes) that greatly influence a gene's potential to undergo regulatory change. These determinants provide a mechanistic basis to serve as a foundation for more-realistic models of gene expression evolution that account for levels of polymorphism and divergence in cis and trans gene regulation.



**Fig. 3.** Mutational variance of gene expression correlates with plasticity of transcriptional response (A) and stochastic noise in protein abundance (B). In each case, the averages of 10 bins of equal sizes are plotted, with error bars denoting two standard errors.

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## Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S6

Tables S1 to S4

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# Gender Disparity in Liver Cancer Due to Sex Differences in MyD88-Dependent IL-6 Production

Willcott E. Naugler,<sup>1,2</sup> Toshiharu Sakurai,<sup>1</sup> Sunhwa Kim,<sup>1</sup> Shin Maeda,<sup>3</sup> KyoungHyun Kim,<sup>1</sup> Ahmed M. Elsharkawy,<sup>1,4</sup> Michael Karin<sup>1\*</sup>

Hepatocellular carcinoma (HCC), the most common liver cancer, occurs mainly in men. Similar gender disparity is seen in mice given a chemical carcinogen, diethylnitrosamine (DEN). DEN administration caused greater increases in serum interleukin-6 (IL-6) concentration in males than it did in females. Furthermore, ablation of IL-6 abolished the gender differences in hepatocarcinogenesis in mice. DEN exposure promoted production of IL-6 in Kupffer cells (KCs) in a manner dependent on the Toll-like receptor adaptor protein MyD88, ablation of which also protected male mice from DEN-induced hepatocarcinogenesis. Estrogen inhibited secretion of IL-6 from KCs exposed to necrotic hepatocytes and reduced circulating concentrations of IL-6 in DEN-treated male mice. We propose that estrogen-mediated inhibition of IL-6 production by KCs reduces liver cancer risk in females, and these findings may be used to prevent HCC in males.

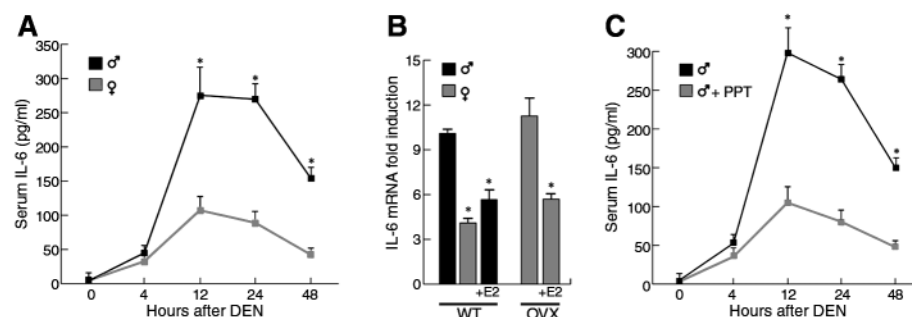
Hepatocellular carcinoma (HCC), the most common primary liver cancer, is a dreaded complication of chronic liver disease that occurs in the setting of risk factors such as hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcoholic liver disease, hemochromatosis, and nonalcoholic steatohepatitis (1). Most HCC appears in cirrhotic livers after years of chronic inflammation. The 5-year survival rate for patients with HCC, the increasing incidence of which is likely due to the spread of HCV (2), is only about 7%. Notably, men are about three to five times more likely to develop HCC than

women (3). A similar or even more pronounced gender disparity is seen in rodent HCC models (4, 5). Furthermore, administration of estrogens

to male mice inhibits development of chemically (DEN)-induced HCC (6). Nonetheless, the mechanisms that account for this gender disparity and the anticarcinogenic activity of estrogens are unknown.

Inflammation is a major contributing factor to carcinogenesis (7). HCC represents a classic case of inflammation-linked cancer (8), and chemically or genetically induced HCC depends on inflammatory signaling (5, 9, 10). To understand the mechanisms underlying gender disparity in HCC, we used the chemical carcinogen diethylnitrosamine (DEN), which causes HCC in 100% of male mice but only in 10 to 30% of female littermates (5, 6). The pathogenesis of HCC in this mouse model differs from that in humans and thus may not be directly comparable to human HCC. Nevertheless, the mouse model of DEN-induced HCC has a histology and genetic signature similar to that of human HCCs with poor prognosis (11) and recapitulates a dependence on inflammation and gender disparity seen in human HCC.

Interleukin-6 (IL-6) is a multifunctional cytokine largely responsible for the hepatic re-



**Fig. 1.** Differential IL-6 production after chemically induced liver injury. **(A)** Concentration of IL-6 in serum of male and female WT mice after injection of DEN (100 mg per kg of body weight;  $n = 3$  mice per time point). **(B)** IL-6 mRNA levels in livers of male, female, or ovariectomized (OVX; ovariectomy was done 2 weeks before DEN administration) female mice 4 hours after DEN injection. E2 (50  $\mu$ g/kg) in corn oil was injected intraperitoneally 2 hours before DEN was administered. **(C)** Male B6 mice ( $n = 3$ ) were injected with ER $\alpha$ -specific agonist propyl-pyrazole-trisphenol (PPT; 5  $\mu$ g/kg in corn oil) 2 hours before DEN injection, and serum IL-6 was measured at the indicated times after DEN injection. Results in (A) to (C) are means  $\pm$  SE. Asterisks indicate a significant ( $P < 0.05$ ; Student's  $t$  test) difference relative to WT male mice.

<sup>1</sup>Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology and Cancer Center, University of California, San Diego, CA 93093, USA. <sup>2</sup>Department of Medicine, Division of Gastroenterology, University of California, San Diego, CA 93093, USA. <sup>3</sup>Division of Gastroenterology, The Institute for Adult Diseases, Asahi Life Foundation, 1-6-1 Marunouchi, Chiyoda-ku, Tokyo 100-0005, Japan. <sup>4</sup>Liver Research Group, University of Newcastle, Newcastle Upon Tyne NE2 4HH, UK.

\*To whom correspondence should be addressed: 9500 Gilman Road, Mail Code 0723 University of California, San Diego, CA 92093-0636, USA. E-mail: karinoffice@ucsd.edu