Caffeine sensitivity in *Drosophila melanogaster*

by

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In loving memory of my grandfather Ziyi Yang,

“Never more than a thought away.”

Abstract

Besides being the most popular drug in the world, caffeine is an attractive tool used in research to help us answer fascinating scientific questions, because caffeine impinges on a number of molecular pathways. In my research, I used caffeine to study DNA repair pathways and insect xenobiotic detoxification.

My work has led to the identification of caffeine-sensitive components of the SMC5/6 complex as critical players conveying resistance to genotoxic stress in *Drosophila*. We were the first group to isolate mutants of *Smc5*, *Smc6* and *MAGE* in *Drosophila melanogaster* and showed that the Smc5/6 complex is not essential for viability but plays a conserved role in protecting against genotoxic agents. Smc5/6 is not required for DNA damage checkpoint response; rather it is involved in homologous recombination repair pathway mediated by Rad51.

My other project was aimed at establishing caffeine as a tool to comprehensively study detoxification responses in *Drosophila*. Using DNA microarray, I measured the transcription response of feeding 8 mM caffeine to wild type larvae, and then derived a set of ~ 48 transcripts that represents a highly significant set of genes affected by caffeine and other xenobiotic treatments such as Phenobarbital (PB), a strong xenobiotic response inducer. Such a condensed "xenobiotic core set" was then used to test whether any of these genes were misregulated in mutants of the JNK or CREB pathways. Because the nature of the Smc5/6 caffeine mutants were not elucidated at the time, I tested whether the caffeine mutants had defects in xenobiotic responses. The ultimate goal was to identify transcription factors that regulate xenobiotic detoxification in *Drosophila*

melanogaster. Another study appeared during my thesis study that showed that *dNrf2/Keap1* is a key regulator of detoxification responses in *Drosophila*. My own analysis did identify this factor too, but also suggested that additional transcriptional regulators likely contribute to the induction of xenobiotic enzymes as well.

Preface

Author Contributions of the Smc5/6 project specifically the following paper:

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Abbreviations

9-1-1	RAD9-RAD1-HUS1
ABC Transporter	ATP-Binding Cassette Transporter
Acetyl CoA	Acetyl Coenzyme A
AChE	Acetylcholinesterase
AMP	Adenosine Monophosphate
aPKC	atypical Protein Kinase C
aRNA	amplified RNA
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATPases	Adenosine Triphosphatase
ATR	Ataxia Telangiectasia and Rad3 related
ATRIP	ATR-interacting protein
BA	Beagle-Accord
BAC	Bacterial Artificial Chromosome
BDSC	Bloomington *Drosophila* Stock Center at Indiana University
Blm	Bloom syndrome, RecQ helicase-like
BP	HMS-Beagle and a P-element
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
bsk	basket
BTB	BR-C, ttk and bab
C. elegans	Caenorhabditis elegans
cAMP	cyclic AMP
CAR	Constitutive Androstane Receptor
cbz	$crebB^{cbz}$
Cdc25	Cell division cycle 25
Cdk1	Cyclin-dependent kinase 1
cDNA	complementary DNA
Chk	Checkpoint Kinase
CL	Cell Lethal
CncC	Cap-and-collar isoform-C
CNS	Central Nervous System

co-IP	co-immunoprecipitated
CREB	cAMP Response Element Binding protein
CyO	Curly of Oster
CYPs	Cytochrome P450s
Dcr	Dicer
DDD	DDT Derivative
DDT	Dichlorodiphenyltrichloroethane
ddt	double double trouble
DGRC	*Drosophila* Genetic Resource Center at Kyoto
DHR96	*Drosophila* Hormone receptor-like in 96
D-jun	*Drosophila* Jun-related antigen
dMRP	Multidrug-Resistance like Protein 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Dependent Protein Kinase
dNTP	deoxynucleotide triphosphates
DSB	Double-Stranded Breaks
DUF	Domain of Unknown Function
EGUF	Eyeless-Gal4 UAS-Flippase
EMS	Ethyl Methane Sulfonate
EST	Expressed Sequence Tag
EtOH	Ethanol
FC	Fold Change
FE4	Fast-E4
FLP	Flippase
FRT	Flippase Recognition Target
GABA	Gamma-Aminobutyric Acid
Gcn5	General control of amino acid synthesis 5
GEO	Gene Expression Omnibus
GFP	Green Fluorescent Protein
GMR	Glass Multiple Response Element
GO	Gene Ontology
GOI	Gene of Interest
GSK3	Glycogen Synthase Kinase-3

GST	Glutathione S-Transferase
HeLa	Helen Lane
hid	Head Inversion Defect
HIV	Human Immunodeficiency Virus
HMDS	Hexamethyldisilizane
HNF4	Hepatocyte nuclear factor 4
hnRNP	heterogeneous nuclear Ribonucleoproteins
hoip	Hoi-polloi
HR	Homologous Recombination
HU	Hydroxyurea
IFCs	Integrated Fluidic Circuits
Inr	Insulin-like receptor
IR	Ionizing Radiation
IRAC	Insecticide Resistance Action Committee
Iso	Isogenized 3rd
IVT	In Vitro Transcription
Jheh1	Juvenile hormone epoxide hydrolase 1
jnj	java no jive
JNK	Jun N-terminal Kinases
Keap1	Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated protein 1
KLF6	Krüppel-Like Factor 6
L3	Third instar
LC50	Lethal concentration 50
MAGE	Melanoma-Associated Antigen
MAPEG	Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism
MBP	Maltose Binding Protein
MDR50	Multi Drug Resistance 50
Mei41	Meiotic mutant 41
MEK	Mitogen-Activated Protein Kinase
MEKK	Mitogen-Activated Protein Kinase Kinase
MF	Morphogenetic Furrow
MMS	Methyl Methane-Sulfonate

MoA	Mode of Action
mod	Modulo
MRN	MRE11–RAD50–NBS1
mRNA	messenger RNA
mRp	Mitocondrial Ribosomal Protein
mTOR	Mammalian Target of Rapamycin
mus	Mutagen Sensitive
nAChR	Nicotinic Acetylcholine Receptor
NaCl	Sodium Chloride
NBS	Nijmegen breakage syndrome
NF	Nuclear Factor
NHEJ	Non-Homologous End Joining Pathway
ninaD	neither inactivation nor afterpotential D
Nrf2	Nuclear factor erythroid 2–related factor 2
Nse	Non-SMC Element
NSE4/EID	Non-Structural maintenance of chromosomes Element 4/EP300-Interacting inhibitor of Differentiation 3
OP	Organophosphorous
P450	Cytochrome P450
PAGE	Polyacrylamide Gel electrophoresis
Par	Paralog
PB	Phenobarbital
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-associated factor
PCR	Polymerase Chain Reaction
PEV	Position-Effect Variegation
PG	Prothoracic Gland
PH3	Phospho Histone H3
phm	phantom
PI3K	Phosphoinositide 3-kinase
PIKK	Phosphatidylinositol 3-kinase-related kinase
PKA	Protein kinase A
POZ	Pox virus and Zinc finger
pre-mRNA	precursor mRNA

PVDF	Polyvinylidene Difluoride
PXR	Pregnane X Rreceptor
qPCR	quantitative Real time Polymerase Chain Reaction
RAD51	Radiation Sensitive51
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNAi	RNA interference
RNApol	RNA polymerase
RNase	Ribonuclease
rp	Ribosomal protein
RRB	rRNA and ribosomal biogenesis
rRNA	ribosomal RNA
RT	Room Temprature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rxn	Reaction
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
S2	Schneider 2
Sb	Stubble
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
shd	shade
SMC	Structural Maintenance of Chromosomes
SMC1/3	Structural Maintenance of Chromosomes1/3 (Cohesin)
SMC2/4	Structural Maintenance of Chromosomes2/4 (Condensin)
SMC5/6	Structural Maintenance of Chromosomes5/6
SpnA	Spindle A
spok	spookier
SR	Serine/arginine-Rich
SRSF2	SR splicing factor
sst	sleepless in seattle
SUMO	Small Ubiquitin-like Modifier
TCGA	The Cancer Genome Atlas

tefu	telomere fusion
TIF-1A	Transcription Initiation Factor 1A
TM3	Third Multiple3
Tor	Target of rapamycin
TORC1	Target of Rapamycin Complex 1
TOPBP1	Topoisomerase-binding protein-1
UAS	Upstream Activating Sequence
UDP	Uridine 5'-Diphospho
UGT	UDP-Glucuronosyltransferase
UV	Ultraviolet
VDRC	Vienna *Drosophila* RNAi Center
WT	Wild Type
wt	weight
YKu70	Yeast Ku Complex 70

Symbols

^{0}	Degree
C	Celsius
CO_2	Carbon Dioxide
ddH_2O	Double-distilled water
g	Gravity
G	Gramme
Gy	Gray=1 J/Kg
h/hr	Hour
Kb	Kilo base
L	Liter
Li	Lithium
M	milli
M	Mole
Ms	Millisecond
N	Nano
Sec	Second
M	Micro (10^{-6})

Chapter 1

Caffeine: Not only a popular drug, but also a versatile research tool

1.1 A brief history of caffeine and overview of its use in scientific research

Caffeine is undoubtedly the world's most popular drug, easily surpassing nicotine and alcohol. It is virtually omnipresent, and not only comes in the form of caffeinated beverages such as coffee, tea and Coca Cola, but also can be purchased as capsules or tablets over the counter. It is the only addictive psychoactive drug that is consumed worldwide, is largely unregulated, sold without a license, and added to beverages that are consumed by children. Caffeine has become such an integral part of people's lives that we do not typically label it as a drug. Yet, there is no doubt that caffeine is a drug, a very potent one indeed.

Coffee, tea, and cocoa comprise some of the most popular drinks in the world; and all of them contain a significant amount of caffeine. Long before realizing the active ingredient is caffeine, people have enjoyed these drinks for the effect of caffeine for centuries. The discovery of coffee is often credited to the romantic tale of Kaldi, an Ethiopian goatherd who lived in the sixth or seventh century, and his dancing goats. Kaldi is said to have noticed that his goats became restless after grazing on the fruits and young twigs of a particular wild bush with glassy green leaves. When Kaldi tasted the fruits himself, the bitterness of the berries deterred him from further chewing the remaining berries, prompting him to throw them into a fire, thus producing the first roasted coffee beans. In another version of this story provided by Antoine Faustus Nairon, a Roman professor of Oriental languages and author of the first printed treaties devoted to coffee in 1671, Kaldi brought the exhilarating berries to an Islamic holy man. The

disapproving holy man threw them into the fire. An unusual and intoxicating aroma arose from the fire and the roasted coffee was discovered (*1*). Another popular source of caffeine uptake is tea. The word "tea" is derived from the Chinese Amoy dialect of the word "t'e,". In China, tea has been around as a prominent drink since its invention in 2700 B.C. According to the legends, the Emperor Shen Nong discovered tea or "Cha" by a simple fortuitous accident. He was boiling some water for drinking, because he had noticed earlier that people who drank boiled water were less likely to fall sick than those who drank directly from a well. A sudden breeze carried a few tealeaves from the branch he used to feed his fire into his pot, into the boiling water. He drank the resulting infusion and, according to the legend, quite enjoyed the stimulating and delicate flavor of the refreshment. Tea was born (*2*).

Following the discovery of coffee and tea and in the early days of their use, coffee and tea were actually considered exclusively as medicine rather than casual drinks. Curiously, chocolate was the first source of caffeine to enter the European bloodstream. Spanish ships carried this native South American product across the Atlantic fifty years before coffee or tea was introduced. The medicinal value of caffeine in promoting wakefulness, improving athletic performances, treating asthma and migraine headaches has been long recognized (*1*).

Caffeine is widely distributed throughout the plant kingdom, has been found in 13 orders of the plants. The most common sources of caffeine are the coffee plant *Coffea arabica*, the tea plant *Camellia sinensis* and the chocolate or cocoa tree *Theobroma cacoa.* Other popular sources are beverages made from

Yerba mate (*Ilex paraguariensis*), guaraná (*Paullinia spp*) and the original Coca Cola was made from cola beans and cola leaves (*Erythroxylum coca*) (*3*).

Why do over 60 plant species make caffeine? Caffeine has evolved as a plant defense mechanism against insect predators and herbivores (*4, 5*). Curiously, members of the genus *Citrus* as well as the related genus *Poncirus* produce caffeine in the male part of the flower, where the amount is toxic to a large number of organisms (*3*). It is thought to be a self-defense strategy to keep the predators away.

Since its discovery in 1819, caffeine has been extensively studied in the context of many biological processes (*6*). Caffeine exerts a plethora of cellular effects which includes i) blocking Adenosine receptor subtype A2A and A1 in the CNS thus promoting wakefulness (*7, 8*), ii) altering intracellular Ca^{2+} levels by promoting Ca^{2+} release through targeting ryanodine receptors in striated and cardiac muscle cells and neuronal cells (*9*) iii) inhibition of cAMP phosphodiesterase therefore activating the cAMP pathway, implicated in memory and learning (*10*) iv) negatively regulating the activities of PI3K-like kinases, which are the key players in cellular responses to DNA damage and acting as a radiosensitizer as well as a G2 checkpoint abrogator (*11*), and v) inducing the expression of a wide range of phase I and phase II detoxification enzymes in insects (*12*). I focused on the last two aspects in my PhD research and will provide a more thorough introduction below.

1.2 Caffeine inhibits ATM/ATR kinases that regulate cell-cycle checkpoints and DNA damage repair pathways

Exogenous sources such as UV radiation, ionizing radiation (IR), hydroxyurea (HU), alkylating agent methyl methane-sulfonate (MMS) and endogenous processes such as spontaneous oxidation, methylation, deamination and depurination of DNA all inflict damages to DNA, which can be detrimental to the cell (*13, 14*). Cells have multiple intertwining pathways that manage DNA damage-surveillance and repair. Checkpoints are regulatory mechanisms that temporarily halt the cell cycle to allow for the completion of critical events such as DNA damage repair, DNA replication, or chromosome segregation. An interesting aspect of caffeine is its ability to sensitize cells to ionizing radiation (IR) and various other DNA-damaging agents by overriding cell cycle checkpoints (*15*). Failure to elicit cell-cycle checkpoints compromises genomic integrity and chromosomal stability. Caffeine blocks checkpoints and thus enhances the sensitivity of cells to ionizing radiation or DNA-damaging drugs, as evidenced by the failure of cells to appropriately sense DNA damage and elevated apoptosis. The presumed mechanism by which caffeine is able to override checkpoint responses is derived from evidence showing that caffeine inhibits the protein kinase activity of Ataxia Telangiectasia-Mutated (ATM) as well as ATM and Rad3 related (ATR) kinases (*11, 16*). ATM and ATR are Phosphoinositide 3-kinase (PI3K)-like kinases that function in DNA damage checkpoints and DNA double strand break (DSB) repair pathways. DSBs are recognized by the Mre11-Rad50-Nbs1 (MRN) complex. Capture of DNA ends by MRN complex rapidly

activates ATM by autophosphorylation that promotes its monomerization and its kinase activity. On the other hand, RAD9–RAD1–HUS1 (also known as 9-1-1) complex and a complex of ATR and its binding partner ATR-interacting protein (ATRIP) are recruited to single-stranded DNA created by incomplete DNA replication. Loading of the 9-1-1 complex brings the ATR activator topoisomerase-binding protein-1 (TOPBP-1) to the damage site. TOPBP-1 binds ATR and activates it in a ATRIP-dependent fashion leading to activation of downstream cascade by phosphorylation (reviewed in *14*). ATM/ATR activation signals downstream signaling cascades that in turn initiate G1, S-phase and G2 DNA damage checkpoints and DNA repair (*17, 18*). Members of the PI3K-like kinases (PIKK) ATM, ATR and DNAPK together coordinate DNA double strand break (DSB) surveillance and repair. ATM/ATR repairs DSB by homologous recombination (HR), whereas DNAPK repairs DSB by a process called nonhomologous end joining (NHEJ) (*17, 18*). Caffeine inhibits ATM, ATR and DNAPK activities with different efficiencies *in vitro* and *in vivo (19, 20)*.

I used *Drosophila* as a model organism to study pathways that are involved in the control of cell-cycle checkpoints or DNA damage repair. By using caffeine as a ATM/ATR inhibitor and hydroxyurea as a DNA damaging agent because it inhibits ribonucleotide reductase (*21*), an enzyme important for new DNA synthesis, I was looking for mutants that are defective in DNA repair or DNA check-point responses that might be in a redundant pathway to ATM/ATR. In a previous ethyl methanesulfonate (EMS) screen for mutants that are sensitive to a mixture of hydroxyurea and caffeine, the Campbell lab identified a mutation

named *huc95E,* which was mainly sensitive to caffeine. Another EMS screen using only caffeine as the selective drug yielded three caffeine-sensitive mutations: *java no jive (jnj*, the original *huc95E*), *sleepless in Seattle (sst)* and *double double trouble* (*ddt*). During my thesis work, I identified *jnj* and *sst* as homologs of two components of the structural maintenance of chromosome (SMC) 5-6 complex, *SMC6* and *MAGE/Nse3* respectively. My hypothesis is that the caffeine-dependent lethality of *jnj* and *sst* mutants is due to impairment in DNA damage responses because the *Drosophila* SMC5-6 complex is involved in ATM/ATR mediated checkpoint and DNA repair. The SMC5-6 complex in yeast and humans is involved in homologous recombination repair of DNA damage and DNA replication (*22-25*). With a collaborative effort, additional alleles of *Smc6*, *MAGE* and *Smc5* were generated. Genomic rescue and qPCR results confirmed that those mutations caused the caffeine sensitivity. Smc6 and MAGE mutants are viable and fertile. *Smc6* and *MAGE* are in the same protein complex. Sensitivity to IR, DNA-damaging drugs, also artificially induced DSB by FLPase, as well as Loss-of-heterozygosity test revealed very mild genome instability of these caffeine sensitive mutants. *Smc6* and *MAGE* mutants are checkpoint competent, however caffeine induces apoptosis in mutant imaginal discs. *Smc6* and *MAGE* genetically interact with ATM/ATR pathway components. Loss of *Rad51,* a key player in homologous recombination repair, rescues caffeine sensitivity in *MAGE* eye specific knockdown. I proposed a two-hit mechanism explaining why caffeine and loss of *Smc5/6* synergistically decrease cell survival (for details see chapter 2 discussion).

1.3 Caffeine serves as a powerful inducer of xenobiotic detoxification responses in arthropods

Xenobiotics are chemical compounds that are not naturally produced by a given organism (such as pesticides, prescription drugs, carcinogens and pollutants) (*26*). Caffeine is a common drug choice in the design of xenobiotic detoxification studies, chiefly because caffeine is a natural and effective pesticide produced by a number of plant species to act as a chemical defense. The first direct evidence that caffeine acts as an insecticide was shown in the tobacco hornworm, *Manduca sexta* larvae (*27*). It was suggested that caffeine acts as an insecticide by inhibiting the production of the secondary messenger cAMP in neuronal tissues through its inhibition of phosphodiesterase. When sprayed onto tomato leaves, an aqueous solution of 2% caffeine was shown to inhibit feeding behavior and kill slugs and orchid snails (*28*). Similarly, treating soil with a 2% caffeine solution caused slugs (*Veronicella cubensis*) to leave the affected area, followed by 100% lethality (*29*). Apart from being an effective molluscicide, caffeine has some anti-fungal properties, acts as a bird repellent and has been used for coyote control (*30-33*). A 2% caffeine solution mimics the natural level of caffeine in coffee seeds (i.e. 0.8-1.8 weight%). Simple 2% caffeine aqueous solutions did not, however, cause lethality in *Drosophila melanogaster* or the berry borer *Hypothenemus hampei (34)*. Instead, caffeine oleate emulsions (a form of activated caffeine by oleic acid and surfactant) showed very high bioactivity (killing in a short time) against both insects (*34*).

The effects of caffeine on gene expression have been studied in vertebrates and *Drosophila* alike. In transcriptional studies, caffeine has induced the expression of well-characterized detoxification genes. In humans, caffeine has been used as a substrate to stimulate the activities of several detoxification enzymes *in vivo* (*35*). In rats, caffeine induces the expression of *CYPA1/A* (a cytochrome P450 gene) and represses *BCL-2* (a tumor suppressor gene) (*36*), however these results were obtained as a result of chronic exposure. In *Drosophila*, feeding caffeine for 4 hours strongly induces a range of Glutathione *S*-transferases (GST) and cytochrome P450 (CYP450) genes (*12*), indicating that caffeine is a potent xenobiotic inducer that can be utilized to model detoxification pathways in flies. These findings were based on a spotted cDNA array that was limited to a selected set of known detoxification genes. Recently, caffeine was shown to induce two P450 genes involved in DDT resistance, *Cyp6a2* and *Cyp6a8*, in a study utilizing a luciferase assay in both SL-2 cell line and adult *Drosophila* (*37*). Moreover, putative binding sites for CREB and AP-1, as well as the presence of barbie-box-like elements (phenobarbital response elements) were predicted in the *Cyp6a8* and *Cyp6g1* promoter region (*38*). *Cyp6a8* is one of the most strongly induced CYP genes by caffeine; overexpression of a single gene, *Cyp6g1,* confers DDT resistance in *Drosophila (39)*. This suggests a possible role for the cAMP and stress signaling MAP kinase pathways in insect detoxification.

For my detoxification project, I carried out a microarray analysis (Affymetrix) to examine the genome-wide response to 8 mM caffeine in

Drosophila wild-type 3rd instar larvae. *Drosophila* larvae increase their size in a very short time getting ready to mature into the adults. Thus, they spend all their time feeding before reaching the proper developmental stage. Supplementing larval media is therefore a very effective way of administrating the drug caffeine to flies, since larvae need to feed in order to grow. This was the first genome wide transcription study of detoxification response in *Drosophila* at the time.

Responsive genes were validated by high throughput microfluidic qPCR. The idea was to use the data from the 8 mM caffeine microarray to derive a set of ~ 48 genes that represents a highly significant set of genes affected by caffeine and other xenobiotic treatments such as Phenobarbital (PB). Such a representative set can then be used to test multiple conditions that would be too expensive to interrogate by microarray analysis or RNA-Seq. For instance, I was interested in the question: which caffeine-responsive genes are under the control of the xenobiotic regulators *dNrf2/Keap1*. Other potential uses of such a condensed "xenobiotic core set" would be to distinguish rapidly induced xenobiotic genes from secondary response genes, by conducting a detailed time course analysis of how caffeine affects these genes. In this work, I used this gene set to test whether any of these genes are misregulated in my caffeine-sensitive mutants. The goal of this approach is to analyze specific mutants for defects in caffeine responses. This is a way to characterize these mutants molecularly. To achieve this, I used a high throughput qPCR-based technology, the BioMark Dynamic array (Fluidigm), that allows the parallel analysis of up to 96 genes in 96 samples in a single 3-hour run (*40*). Because multiple binding sites of AP-1 (the downstream effector of JNK

signaling) and CREB were found in the promoter region of *Cyp6a2* and *Cyp6a8,* P450 enzymes that are associated with DDT resistance (*41-43*), I also tested a heat-shock driven dominant negative *cbz* mutant (*Drosophila CREB*), a heat-shock driven dominant negative *basket* (*Drosophila JNK*) using the above-mentioned set of 48 genes (*44-47*). To examine whether one of the *Drosophila* detoxification regulators, *dNrf2* (*48*), played a role in caffeine-mediated responses, the genes unique to the caffeine response were tested in *dNrf2*/*Keap1* loss-of-function and gain-of-function mutants (*49*). NRF2 has been mainly studied for its regulation of phase II enzymes in mice and cell lines (*50, 51*), thus it is possible that *dNrf2* is not the only transcription factor required for the induction of detoxification. Thus, the ultimate goal of my project was to identify hitherto unknown transcription regulators of xenobiotic responses in insects. While my approach would have independently identified *dNrf2/Keap1* (a paper was published during my thesis that identified this factor as a regulator of xenobiotic detoxification in *Drosophila*) because Keap1 was indeed a highly induced gene in my microarray, I did find that other putative transcription regulators that were differentially expressed in response to caffeine. The top repressed gene was an Acyl-CoA N-acyltransferase *CG15155* and another Acyl-CoA N-acyltransferase *Gcn5 ortholog* was found to be significantly caffeine-induced independent of *dNrf2/Keap1* signaling, drawing our attention to investigate in the future the possible link between these histone acetyl transferases and caffeine-induced detoxification gene expression.

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Chapter 2

The Smc5/6 complex confers resistance to caffeine and genotoxic stress in *Drosophila melanogaster*

2.1 Introduction:

The evolutionarily conserved Structural Maintenance of Chromosomes proteins are essential for the organization, segregation, and stability of the genome (*1-3*). The SMC proteins are found in all kingdoms of life from bacteria to humans. In bacteria, there is only one gene encoding a single SMC protein. In eukaryotes, three functionally distinct SMC complexes have been defined: SMC1/3 (cohesin), SMC2/4 (condensin), and the otherwise unnamed SMC5/6 complex, each with a unique set of regulatory subunits (Figure 2.1). Cohesin holds sister chromatids together after DNA replication and plays important roles in regulation of gene expression and DNA repair (*4*), while condensin is essential for mitotic chromosome organization and segregation (*5*). The lesser know Smc5/6 is mainly thought to be involved in homology based DNA maintenance at highly repetitive regions and during meiosis. Specifically, the cohesin complex establishes the cohesion of sister chromatids during mitosis (*6*), and likely to have a role in transcription activation via DNA binding (*7, 8*). Condensin promotes chromosome condensation to ensure accurate chromosome segregation during mitosis (*9*). Besides these fundamental roles cohesin and condensin have in regulating chromosome metabolism, they are also important for DNA damage responses (reviewed in (*2*)). The three SMC complexes belong to a large family of ABC type ATPases that also includes Rad50 of the MRN complex, a key player in DNA damage response. Both cohesin and condensin are involved in checkpoint activation. Cohesin is involved in homologous recombination (HR) repair of DNA double-stranded breaks (DSB) (*10-12*), whereas condensin is required for repair

of single-stranded or double-stranded DNA breaks depending on the subtype of condensin in human cells (*13, 14*). The SMC5/6 complex is less well characterized but is primarily required for several HR based cellular processes, including DNA repair of DSB, restart of collapsed replication fork, ribosomal DNA maintenance, telomere elongation, and chromosome dynamics during meiosis (*15-18*).

One of the most extensively described functions of SMC5/6 is DNA DSB repair via HR. Mutants of SMC5/6 are sensitive to crosslinking agent UV, ionizing radiation, alkylating agent methyl methane-sulfonate, topoisomerase I inhibitor camptothecin, and replication blocker hydroxyurea. SMC5/6 is recruited to ectopically induced DSB sites as shown in *S. cerevisiae* and human cells by chromatin immunoprecipitation experiments (*19, 20*). In yeasts, nematodes, chicken DT40 cells, plants and human cells, SMC5/6 facilitates DNA repair through inter-sister chromatid recombinational repair (*19-24*).

The clue that the Smc5/6 complex is involved in DNA repair came before the discovery of this complex in fission yeast. In *Schizosaccharomyces pombe*, smc6 was first identified as Rad18 in a screen for mutants sensitive to radiation (*25-27*). Subsequently, smc5 and other Non-SMC element (Nse) subunits of the complex were discovered, mutants of which are also sensitive to various DNA damaging agents (*16, 28, 29*). The Smc5/6 complex in the yeasts is made up of eight subunits that form three sub-complexes: Smc6-Smc5-Nse2, Nse1-Nse3-Nse4, and Nse5-Nse6. Smc5 and Smc6 dimerization occurs through their hinge regions thus forming the core. Nse2/MMS21 is a SUMO ligase that binds only to

Smc5. In different systems, Nse2 SUMOylates various targets that include not only components of the SMC5/6 complex such as Smc5, Smc6, Nse3-4 and itself, but also YKu70 of the non-homologous end joining pathway (NHEJ), cohesion subunits Scc1 and SA2 and many other telomere associated proteins (*30, 31*). Nse1 and Nse 3 interact with Nse4, the kleisin component of the complex, to form a sub-complex that bridges the head domain of the Smc5-Smc6 heterodimer. Nse1 has a RING domain that functions as an E3 ubiquitin ligase (*32, 33*). Nse3 is of particular interest because of its homology with mammalian MAGE (melanoma-associated antigen) proteins, which form an expanded family of over 50 members that share a 200 amino acid protein-protein interaction domain, classified into two types (*34*). MAGE proteins of Type I were identified because they are highly expressed in human melanoma cancer patients, and may play a role in resistance to chemotherapeutic agents (*35*). In fact, 85% of cancer cell lines over-express at least one Type I MAGE gene (*34*). In contrast, Type II MAGE genes, such as Necdin, MAGEL2, and MAGED1, are expressed in normal tissues and have important roles in mammalian development (*36-38*). MAGEG1 was identified in the human SMC5/6 complex. The crystal structure of MAGEG1 reveals its interaction with RING protein Nse1 and this interaction stimulates the ubiquitin ligase activity of Nse1 (*29, 39*) The MAGEG1/Nse3 and Nse4/EID interaction is also conserved in human cells (*40*). Some, though not all, MAGE proteins contribute to SMC5/6 complexes like those described in yeast, suggesting a conserved role of MAGE proteins as part of distinct Smc5/6 complexes in chromosome maintenance (*29, 39-43*). It appears that peptide modifications of

chromatin-associated proteins are important for SMC5/6-mediated DNA damage response mechanisms and cross talk between HR and NHEJ pathways.

In budding yeast, all the known Smc5/6 associated proteins are encoded by essential genes that function in DNA replication, recombinational DNA repair, and chromosome segregation (*32, 44, 45*). In fission yeast, Nse5 and Nse6 mutants are viable with defects in repairing DNA damages induced by UV or alkylating agent MMS (*46*). There is no conservation of the Nse5 and Nse6 protein sequences between the two yeasts. In *Xenopus*, *C. elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, Chicken DT40 cells and human cell lines, only Nse1-4 were identified, suggesting Nse5-6 are yeast specific. Epistasis experiments in yeasts and vertebrate cells have placed *Smc5/6* genes in the homologous recombination-based DNA repair pathway that involves Rad51 nucleofilament proteins (*47*). The *Drosophila melanogaster* genome encodes unique homologs of *Nse1-4*, *Smc5* and *Smc6*, making this an attractive system for defining conserved molecular mechanisms of Smc5/6 complex. In *Drosophila*, Smc5/6 plays a role in maintaining genome stability in heterochromatin regions by repressing non-sister chromosome recombination events (*18, 47*). *Drosophila* Smc5/6 also serves a conserved molecular role in blocking Rad51 loading during this process and compromising Smc6 activity in S2 cells caused chromosome defects, suggesting Smc5/6 functions are essential (*47*). Regulation of homologous recombination-mediated DSB repair largely relies on two kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR). ATM and ATR are phosphoinositide 3-kinase-like kinases (PIKK) that are

activated by double strand breaks, turning on a network of DNA damage response signaling pathways that coordinate cell cycle progression and DNA repair (*48*).

Caffeine is a PIKK inhibitor commonly used to inhibit ATM and ATR kinase (*49*). We sought to identify novel genes functioning in DNA damage response pathways that are redundant with ATM and ATR, by screening for conditional eye phenotypes in adult flies that were fed caffeine throughout larval development. We found unexpectedly that three *Drosophila* genes, *Smc5*, *Smc6* (CG5524) and *MAGE* (CG10059), are not essential under normal growth conditions, but are required for resistance to caffeine exposure throughout development. Interestingly, these mutants are also hypersensitive to genotoxic agents, suggesting a conserved role for the Smc5/6 in DNA damage repair. Caffeine induces apoptosis in the mutant flies in a process mediated by ATM and ATR that does not involve conventional cell cycle checkpoints. We have thus identified a novel caffeine-sensitive mechanism that prevents apoptosis in proliferating cells exposed to genotoxic stress. These findings provide new insight into Smc5/6 functions that were previously shown to prevent apoptosis in human cells (*42*).

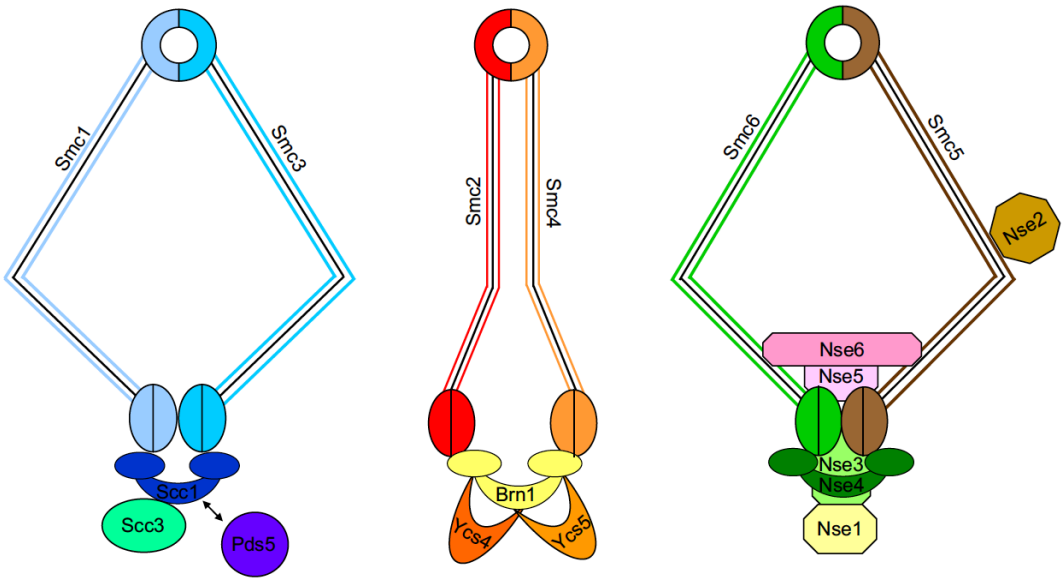

Figure 2.1 Schematics of cohesion (Smc1/3), condensin (Smc2/4) and Smc5/6 complex in budding yeast, adopted from (*50*). Cohesin holds sister chromatins together facilitating successful segregation and faithful DNA repair by homologous recombination. Scc3 and the kleisin subunit Scc1 interact with the ATPase head region of Smc1 and Smc3 forming a ring-like structure. Pds5 is one of the factors interacting with chromatin-bound cohesin to modulate its association with chromatin. Condensin is associated with 3-D organization and segregation of chromosomes during cell divisions. The kleisin subunit Brn 1 bridges the globular ATPase head domains of Smc2 and Smc4 and interacts with subunits Ycs4/Ycs5, which contains HEAT-repeats that binds directly to DNA. Smc5/6 is largely involved in homologous recombination based DNA repair. Nse5-6 are yeast specific subunits, seems not present in higher organisms. Kleisin subunit Nse 4 interacts with Nse1 and Nse3 (MAGE) and ATPase head domain of Smc5 and Smc6. Smc5-associated Nse2 (MMS21) is a SUMOlyase that is required to respond to DNA damage.

2.2 Materials and Methods

***Drosophila* stocks and husbandry**. All crosses were carried out at 25°C, and flies were maintained on media formulated at the Bloomington *Drosophila* Stock Center at Indiana University (BDSC) with *p*-Hydroxy-benzoic acid methyl ester or propionic acid as the fungicide. Stocks were obtained from the BDSC, the Vienna *Drosophila* RNAi Center (VDRC), or the *Drosophila* Genetic Resource Center at Kyoto (DGRC) or generated in our laboratories where specified. Fly stocks used were:

y^1 w; P{70FLP}(51)11 P{70I-SceI}2B sna^{Sco}/CyO, S2*

*w^{1118}; P{70FLP}*10; *Sb^1/TM6, Ubx*

y^1 w^{67c23} P{Crey}1b; D/TM3, Sb^1*

P{GawB}NP2592

w; Dr^1/TMS, P{Delta2-3}99B*

P{GSV1}GS3245

P{GSV6}GS14577

P{ey3.5-GAL4.Exel}2

C(1)DX, y[1] f[1] / w[1] mei-41[D3]

UAS-ATR-RNAi

UAS-ATM-RNAi

UAS-NBS1-RNAi

UAS-SpnA-RNAi

UAS-MAGE-RNAi/CyO (TRiP)

Ethyl methanesulfonate (EMS) screen for caffeine-sensitive mutants on chromosome 3R.

The isogenized fly stock *FRT82B* carries a transgenic Flippase Recognition Target (FRT) site inserted at polytene segment 82B on chromosome 3R and was used to screen for caffeine sensitivity. Adult male flies were mutagenized by feeding with 15 mM EMS dissolved in 1% sucrose for 12 h. After a one day recovery period, mutagenized males were crossed to *EGUF; FRT82B GMR-hid, CL/TM3, Sb* virgin females. Three to five F1 progeny *EGUF/+; FRT82B/FRT82B GMR-hid, CL* males with normal eye morphology were crossed

to *EGUF; FRT82B GMR-hid, CL/TM3, Sb* virgin females. The F2 progeny were raised in media with 2 mM caffeine. Individual male non-balancer F2 flies displaying abnormal eye morphology in both eyes were backcrossed to *EGUF; FRT82B GMR-hid, CL/TM3, Sb* virgin females, and the F3 progeny were raised in media without caffeine to identify any flies with caffeine-independent eye defects. Once the caffeine-dependence of the eye phenotype was confirmed, each mutation was tested by complementation with the original jnj^{huc95E} allele (*52*) or mapped using the *Drosophila* 3R deficiency kit (BDSC). Both the jnj^{R1} and sst^{RZ} lines emerged from this screen.

Sequencing of candidate genes. Targeted re-sequencing of mapped caffeine-sensitive loci was used to identify mutations in candidate genes. Genomic DNA from 50 adult flies was extracted using DNAzol reagent (Invitrogen, Burlington, ON, Canada). Overlapping PCR fragments about 10 kb in size were amplified using a Long Range PCR kit (Invitrogen). These fragments covered each region predicted to contain a mutation and 10 kb on either side. The PCR products were sequenced using Illumina technology and data was analyzed with Bowtie software (Illumina Inc., San Diego, CA) (*53*). Mutations were confirmed by Sanger sequencing with BigDye v3.1 (Applied Biosystems, Carlsbad, CA). Restriction digestion (BpmI) of a genomic PCR fragment was used to confirm the mutation in jnj^{R1}.

*** Generation of the *MAGE* allele sst^{XL} using gene targeting**. The "ends-out" method (*54*) was used to produce a targeted deletion of *MAGE.* Specifically, 3 kb genomic regions upstream and downstream of the *MAGE* genomic locus were

* This experiment were solely done by Xiao Li.

amplified by PCR from a *Drosophila* BAC clone (BACPAC Resources Center, RP98-3E11), using the following PCR primers

5'-ATTCATGCGGCCGCCGAAACTCAAACGCAGCGAA and

5'-ATTCTAGGTACCGAGAAGTGCTAGCCATTTCGAG or

5'-ATTCTAGGCGCGCCGGAGTAAACGCGGAGTAGAATACC and

5'-ATTCATCGTACGGGAAGGGGATCAGGATTGAA.

The two PCR fragments were subcloned into the notI-kpnI (Acc65I) or AscI-BsiWI sites of the ends-out vector *P[w25.2]* to produce a donor construct *P[w25.2]_NK_AB*. Seven transgenic lines were generated by P element transformation of a w^{1118} strain using *P[w25.2]_NK_AB* (BestGene Inc, Chino Hills. CA). The three lines in which the *P[w25.2]_NK_AB* was located on chromosome 2 were tested for efficient excision by crossing to a line carrying the *FLP* recombinase (w^{1118}*; P{ry+t7.2 70FLP}10;* Sb^1*/TM6, Ubx*). One of the three transgenic lines (*6030-1-6M*) with the highest excision efficiency was chosen as the donor line, and crossed to y^1 *w*; P{70FLP}11 P{70I-SceI}2B* sna^{Sco}*/CyO, S2* (BDSC #6934). The parents were allowed to lay eggs for two days in a vial, and on the third day the larvae were heat-shocked for 1 h in a 38°C water bath. F1 virgin females were collected and crossed to w^{1118}; *P{70FLP}*10; Sb^1*/TM6*, *Ubx* (BDSC #6938) males. About 100 F2 progeny were selected by screening for nonwhite flies from about 1000 independent crosses. Each of these progeny was crossed to w^{1118}; *P{70FLP}*10; Sb^1*/TM6*, *Ubx* to make stocks. Twenty five independent lines were identified that exhibited correct targeting as detected by PCR of genomic DNA and loss of MAGE protein expression by immunoblotting

with a guinea-pig anti-MAGE antibody (*55*). The *white* marker of these lines was removed by crossing to a line carrying a Cre recombinase (y^1 w^{67c23} *P{Crey}1b; D*/TM3,* Sb^1 (BDSC #851). The resulting lines were tested for heterozygote and homozygote viability under normal conditions, yielding the line named sst^{XL}.

Generation of a genomic rescue construct for *MAGE* on chromosome 2. Genomic DNA was isolated from the isogenized strain P{ry[+t7.2]=neoFRT}82B to PCR amplify (Sequal Prep Long PCR Kit, Invitrogen) a 4 kb fragment spanning from 3 kb upstream of the MAGE gene (genomic locus 3R:2,983,898, based on the predicted transcription start site), to 206 bp downstream MAGE stop codon (genomic locus 3R: 2979891). The PCR product was digested with the restriction enzyme xbaI and cloned into the pCasper-hs-act vector. Transgenic flies were generated by BestGene Inc.

*** Generation of additional *Smc6* alleles by P-element mediated excision.** The *Smc6* deletion allele jnj^{X1} was generated by imprecise excision of a P element in *P{GawB}NP2592* (DGRC #104251). This insertion, hereafter referred to as *NP2592*, is located 7 bp upstream of the putative transcriptional initiation site of *CG5524* (*Smc6*) (3R:20,014,770..20,019,145). Its location was confirmed by genomic PCR using primers flanking the *NP2592* locus. To excise out *NP2592*, *NP2592* virgin females were crossed to w*; Dr^1*/TMS, P{Delta2-3}99B* (BDSC #1610) males carrying a Δ2-3 transposase. Single virgin F1 females of genotype *ΔNP2592/ TMS,{Δ2-3}99B* were crossed to *Ly/TM3, Sb* males. Single F2 males of genotype *ΔNP2592/TM3, Sb* were crossed to virgin *Ly/TM3, Sb* virgin females to establish balanced lines. About 200 candidate lines were produced and

* These experiments were solely done by Xiao Li.

subsequently tested for sensitivity to 2 mM caffeine. Six lines were found to be homozygous viable but caffeine-dependent lethal. Genomic PCR was used to confirm that there were deletions around the original P insertion sites in these stocks. One of the resulting lines was renamed jnj^{XL}.

*** Molecular characterization of *Smc5* alleles.** The location of *P{GSV1}GS3245* (BDSC #200582) and *P{GSV6}GS14577* (BDSC #205862) within coding exon 2 of the *Smc5* gene was confirmed by genomic PCR using primers 5'-CGTTTCCACGATTTGTTACTGACA and 5'-CGTTTTTGCTTCTTAACCAGATCAC. These lines were renamed $Smc5^{P5}$ and $Smc5^{P7}$, respectively. *Df(3L)BSC418* (BDSC #24922) is a sequence mapped chromosome deletion (78C9;78E1) that includes the *Smc5* locus and nearby genes.

Embryo collection, drug administration and *ionizing radiation (IR) treatment. Parental flies were allowed to lay eggs in collection cages on apple juice or grape juice agar plates with yeast paste for 20 h. The eggs were gently removed from the agar plates using distilled water and a brush and collected using a small cloth-bottomed basket, and then arrayed on new apple juice agar plates. For each drug or radiation treatment, at least 100 embryos were transferred with a thin layer of agar underneath into each of 3 vials containing medium. Drug stocks were pre-added into the media to the appropriate working concentration, with the exception of methyl methanesulfonate, which was added into the medium 48 hours after transferring the embryos. For drugs dissolved in DMSO, an equal amount of DMSO alone was added into medium fed to control flies. The

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following drugs were used: caffeine (Sigma-Aldrich, St. Louis, MO, stock 1 M in water, final concentration 0.25-2 mM); camptothecin (Sigma-Aldrich, stock 12.5 mM in DMSO, final concentration 0.025 mM), methyl methanesulfonate (Sigma-Aldrich, stock 99%, final concentration 0.005-0.015%) and HU (Sigma-Aldrich, stock 1 M, final concentration 4-8 mM). For IR, third instar larvae were irradiated at doses of 20 and 40 Gray using an irradiator (Gammacell 220–Cobalt-60, Atomic Energy of Canada, 1979). The survival index (*p*) of a given genotype was calculated by dividing the number of adult survivors of the genotype resulting from media with a given reagent concentration or treatment (*n*) by the number of adult survivors of the same genotype resulting from media without aforementioned reagent or treatment (*N*).

***Immunoblotting.** For each sample, ten 3-4 day-old adult flies were collected, frozen in liquid nitrogen and homogenized using a pestle in a 1.5 ml eppendorf tube. Mild lysis buffer (50 mM Tris, 150 mM NaCl, and 1% Triton X-100, pH 8.0) was then added (10 µl per fly) to solubilize the tissue. The suspension was centrifuged at 20,000*g* for 10 min. at 4°C and the supernatant was mixed and boiled with 2X Laemmli Buffer. Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes for immunoblotting. A 1:2500 dilution of guinea pig anti-MAGE serum was used to detect MAGE protein (*55*).

Genetic interactions of *ATM*, *ATR, NBS1* and *RAD51* loss-of-function with *MAGE* and *Smc6*. Double mutants of *ATR* and *Smc6* were generated using fly stock $mei\text{-}41^{D3}$ (*56*) and *Smc6* alleles jnj^{X1} *and* $jnj^{Df(3R)Exel6198}$. Knockdown of *ATM*, *ATR* or *NBS1* function in *MAGE* or *Smc6* homozygous mutant eye clones

was achieved using the *EGUF* system, which uses the *eyeless-Gal4* driver to express transgenes throughout eye development (*57*). The *EGUF* system also ensures that all ommatidia of the adult eye are homozygous for either *Smc6* or *MAGE* mutant alleles, because of an eye-specific *GMR-hid* transgene that eliminates non-mutant ommatidia. RNAi knockdown of *MAGE* alone or double RNAi of *MAGE* and *Rad51* ortholog *SpnA* in the eye was achieved by crossing appropriate RNAi constructs containing males to *UAS-Dcr2/CyO; ey-Gal4/TM3,Ser* virgin females. For each genotype, five to nine specimens were photographed, and representative phenotypes are shown.

*** cDNA clones, Cell culture, transfections, and co-immunoprecipitation**. Full-length cDNA clones for *Nse1* (GM14348) and *Nse4* (IP09347) were obtained from the Canadian *Drosophila* Microarray Centre. The *MAGE* (RE25453) clone was obtained from the *Drosophila* Genomics Resource Center (DGRC, Indiana University). *Drosophila* S2 cells were grown at 25°C in TNM-FH medium (SH30280.02, Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum. Expression constructs for transfection of S2 cells were created by inserting relevant full-length coding sequences into the *Drosophila* Gateway destination vectors (obtained from the DGRC). S2 cells were transfected with relevant expression constructs using dimethyldioctadecyl-ammonium (*58*). Cells were harvested 24 h after transfection, washed once in phosphate buffered saline, (pH 7.2), and re-suspended in the mild lysis buffer supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The lysate was centrifuged for 10 min. at 20,000*g* at 4°C, and the supernatant transferred to a

* These experiments were solely done by Xiao Li.

fresh tube. 200 µl of supernatant was mixed with 20 µl of protein G agarose beads (GE Healthcare Life Sciences, Piscataway, NJ) pre-bound with 5 µg of antibody in 800 µl mild lysis buffer. The agarose beads were then incubated for 1 h at 4°C with rocking, washed six times using mild lysis buffer and the bound proteins analyzed on immunoblots.

***In vitro* pulldown assays**. pMBP-MAGE was previously described (*55*) and the control pMBP construct was supplied with a Maltose binding protein (MBP) purification kit (New England Biolabs, Ipswich, MA). Expression constructs were produced by inserting relevant full-length coding sequences into a Gateway pDEST-14 expression vector. MBP fused MAGE (MBP-MAGE) was expressed in *Escherichia coli* (ER2523, New England Biolabs) and immobilized onto amylose resin (E8200S) according to the manufacturer's directions. ^{35}S labeled probe proteins were expressed from Gateway pDest14 vectors using the TNT-coupled *in vitro* transcription-translation system (Promega, Madison, WI). For the *in vitro* binding assay, ^{35}S-labeled probe proteins were incubated with immobilized MBP-MAGE proteins in 500 µl of buffer (20 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1% Tween-20, pH 7.6) containing 0.25% bovine serum albumin (BSA) and protease inhibitor cocktail (*59*) overnight at 4°C with end-over-end mixing. The resin was washed six times in 500 µl of the same buffer, and the bound proteins were resolved by SDS-PAGE and detected by autoradiography.

Scanning electron microscopy (SEM) and immunohistochemistry. Adult heads were prepared for SEM according to the HMDS method described in

Drosophila Protocols (*60*) and iMAGEd using a Scanning Electron Microscope (FEI (XL30), Philips, Hillsboro, OR). Dissection, fixation, BrdU labeling, and antibody staining of third larval instar eye-antennal discs were also carried out as described in *Drosophila Protocols.* Antibodies for immunohistochemistry included anti-cleaved caspase 3 (1:1600 dilution, Cell Signaling Technologies, Beverly, MA), anti-BrdU (1:200 dilution, Pharmingen San Jose, CA), and anti-phospho-histone H3 (Cell Signaling, 1:1000 dilution). Secondary antibodies were used at a dilution of 1:1000 (Alexa Fluor 488 and 586, Invitrogen). For the detection of apoptosis in third instar imaginal discs with an anti-cleaved caspase 3 antibody, embryos were collected at one hour intervals on grape juice plates and larvae were reared on yeast paste plates until the L3 molt. They were then transferred to 2 mM caffeine medium 32 h after the L3 molt and allowed to develop for a further 12 h before dissection. IMAGEs of the dissected discs were acquired using a LSM 700 confocal microscope (Carl Zeiss Inc., Thornwood, NY) and processed using Zen (Carl Zeiss). A maximum projection of all stacks of a confocal iMAGE was used to quantify the signal intensity of staining using a lower threshold to eliminate background staining. This value was divided by the area of each eye disc to obtain a ratio representing the relative amount of immunostaining. Data represent at least 7 eye discs per genotype per treatment.

Quantitative RT-PCR. Total RNA was extracted from adult flies using Trizol reagent (Invitrogen). RNA concentration and integrity were determined by a Nanodrop ND-1000 (NanoDrop products, Wilmington, DE) and Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA), respectively. One μg of

total RNA per reaction was used for double strand cDNA synthesis (Applied Biosystems). Then, 2.5 μl of 1/20 diluted cDNA was used for each qPCR reaction with quantification based on SYBR Green incorporation (Applied Biosystems).

2.3 Results

2.3.1 A screen for caffeine-sensitive eye mutants reveals three loci on chromosome 3R

The compound eyes of *Drosophila* are ideal tissues to detect defects in proliferation and apoptosis as they are not essential for survival, but they are sensitive to developmental perturbations and easy to score for mutant phenotypes. To identify novel genes functioning in DNA damage response pathways that are redundant with ATM and ATR, we previously performed a genetic screen to identify conditional eye phenotypes in adult flies fed 2 mM caffeine and 3 mM hydroxyurea (HU) throughout larval development (*52*). While caffeine inhibits ATM and ATR, HU inhibits dNTP production, stalling ongoing DNA replication and generating single strand or double strand DNA breaks, thereby activating DNA damage responses regulated by ATM and ATR. At the drug concentrations used, there were no phenotypic effects in wildtype flies. In this screen, we used the "*EGUF, GMR-hid*" (*EGUF*) system to produce homozygous mutant clonal cells in the entire adult eye of an otherwise heterozygous fly (*57*). This screen identified a single caffeine-sensitive locus (*huc95E*) on chromosome arm 3R, here renamed *java no jive* (*jnj*), which we mapped to cytological region 95E by complementation testing with chromosomal deficiencies (*52*). Hemizygous *jnj* eye

mutants exhibit caffeine-dependent small, rough eyes associated with increased apoptosis.

To identify novel DNA damage pathway components, we have now carried out a new screen of chromosome arm 3R for conditional caffeine-sensitive eye phenotypes. By screening 9098 males, we identified three loci on chromosome arm 3R including six additional alleles of *jnj*, two mutant alleles of a locus called *sleepless in seattle* (*sst*), and one allele of a novel locus called *double double trouble* (*ddt*), that has not yet been linked to a specific gene (Figure 2.2, Figure 2.3A). All hemizygous *jnj, sst* and *ddt* mutants exhibit caffeine-dependent pupal lethality (Figure 2.2.2B-D and data not shown).

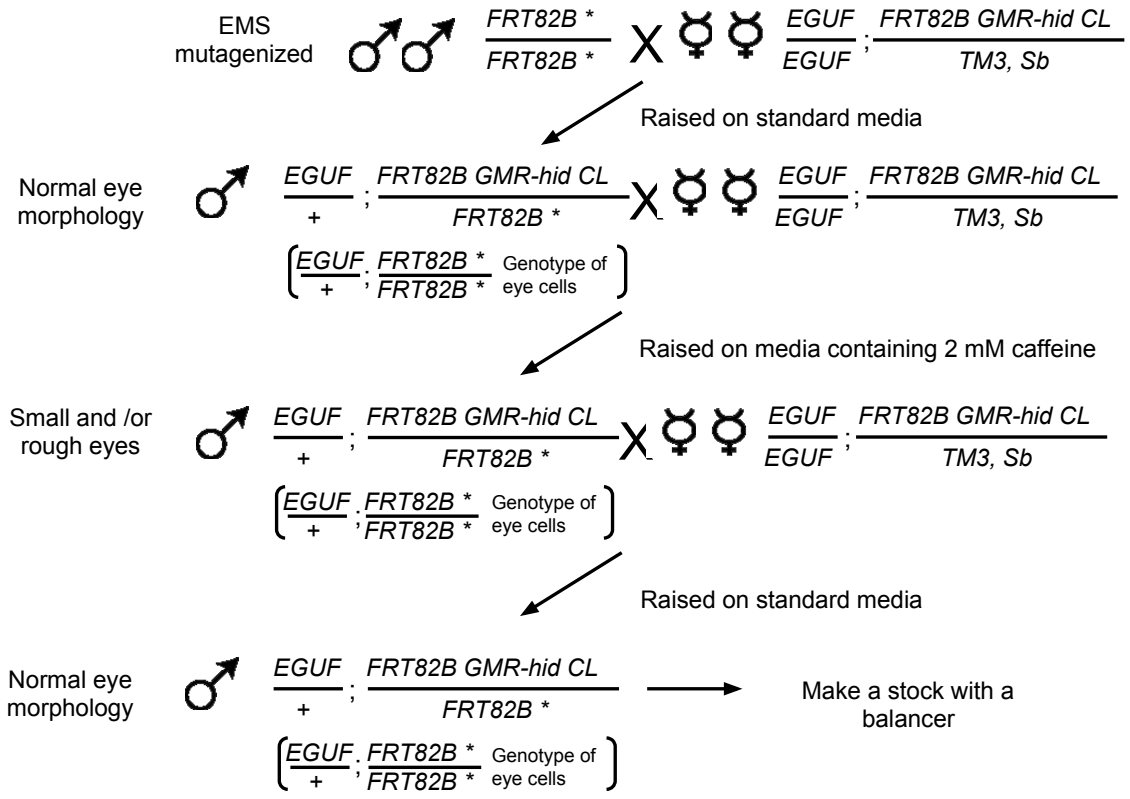


Figure 2.2 An ethyl methanesulfonate (EMS) screen for caffeine-sensitive mutants on chromosome 3R.
EMS mutagenized males carrying transgenic *FRT82B* sites were then crossed en masse to *y,w; EGUF; FRT82B GMR-hid/TM3, Sb* virgin females in standard molasses and cornmeal media at 25^{o}C. Non-TM3, Sb progeny males containing normal looking eyes were then collected and

crossed in pools of 3-5 males to 3-5 *y,w; EGUF; FRT82B GMR-hid/TM3, Sb* virgin females in media containing caffeine, and Non-TM3, Sb progeny males containing developmental defects in both eyes were selected and individually tested with *y,w; EGUF; FRT82B GMR-hid/TM3, Sb* virgin females in normal media to eliminate any false positive caffeine independent mutations that might have arisen in the male germline. Once a caffeine dependent phenotype was confirmed, the mutant was crossed to *y,w; EGUF; FRT82B GMR-hid/TM3, Sb* females to establish a balanced stock. "*" indicates a putative mutation. Experiments done by ST and RZ.

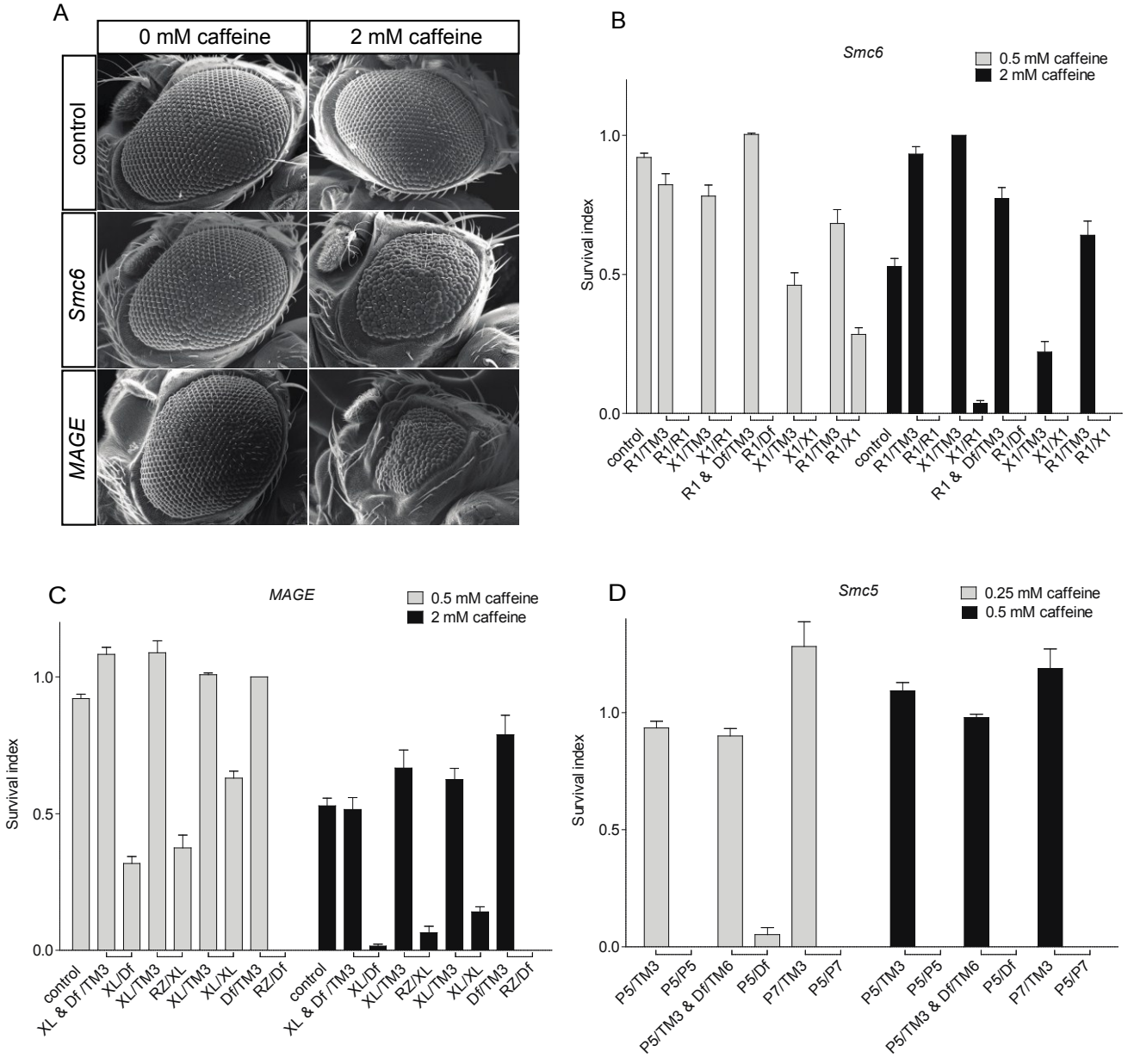


Figure 2.3 Eye phenotypes in caffeine-sensitive mutant flies.
(A) Caffeine-dependent eye phenotype of *Smc6* and *MAGE* mutants. Fly genotypes are as follows. Control: *EGUF/+; FRT82B +/FRT82B GMR-hid*. *Smc6* (loss of *Smc6* in eye cells): *EGUF/+; FRT82B jnj^{R1}/FRT82B GMR-hid. MAGE* (loss of *MAGE* in eye cells): *EGUF/+; FRT82B sst^{RZ}/FRT82B GMR-hid*. (B-D) *Smc6, MAGE* or *Smc5* homozygous, trans-heterozygous or hemizygous mutants have reduced survival when raised in media with caffeine. Bars represent the survival index (*p*) and error bars represent SEM. "␣" indicates flies eclosed from the same cross. Absence of a bar indicates no surviving flies. Wildtype control flies are *w^{1118}*. (B) *Smc6* mutants

are sensitive to caffeine. *R1* (jnj^{R1}) is an allele from the caffeine screen, *X1* (jnj^{X1}) was generated by an imprecise excision of a P-element adjacent to the 5'UTR of *Smc6*, and *Df* (*Df(3R)Exel6198*) is a deficiency chromosome uncovering the *Smc6* locus. (C) *MAGE* mutants are sensitive to caffeine. *RZ* (sst^{RZ}) is an allele from the caffeine screen, *XL* (sst^{XL}) is a targeted knockout, and *Df* ($Df(3R)Antp^{1}$) is a deficiency chromosome uncovering the *MAGE* locus. (D) *Smc5* mutants are sensitive to caffeine. Both *P5* ($Smc5^{P\{GSV1\}GS3245}$) and *P7* ($Smc5^{P\{GSV6\}GS14577}$) contain P-element insertions in a coding exon of *Smc5*, and *Df* (*Df(3L)BSC418*) is a deficiency chromosome uncovering the *Smc5* locus. (A) is done by RZ, (B,C,D)Done by RZ and repeated by XL .

2.3.2 Mutations in *Smc6* cause caffeine-dependent defects in *java no jive* mutant flies

Deletion mapping indicated that all of the caffeine-sensitive *jnj* alleles were viable in hemizygous combinations with deletions uncovering region 95E, indicating that the homozygous lethality of most *jnj* alleles was caused by second site mutation(s). Homozygotes for one allele, jnj^{R1}, were viable on regular media, but died at the pupal stage when raised in media containing caffeine (Figure 2.3B). Sequencing of candidate genes in the *jnj* region identified a four base pair deletion in exon two of the FlyBase annotated gene *CG5524* (del_ATCT at position 334-337 bp from the presumptive start codon), creating a frameshift resulting in a stop codon at position 133 of the presumptive 1122 amino acid protein (Figure 2.4A). The predicted CG5524 protein has highest amino acid identity with SMC6 (Structural Maintenance of Chromosomes 6) in other species. SMC6 regulates chromosome stability in yeasts (*16-18*), and is implicated in heterochromatic DNA repair in *Drosophila* (*47*). We tested *CG5524* (hereafter called *Smc6*) and four neighboring genes for levels of expression by quantitative RT-PCR of mRNA from whole flies. Levels of *Smc6* mRNA were greatly reduced with all seven alleles of *jnj*, ranging from 9% to 24% of control levels (Figure 2.5A) whereas nearby genes showed little change in expression. Despite extensive

sequencing efforts, we were not able to identify the nature of *jnj* alleles other than jnj^{R1}, suggesting that these unmapped mutations reside in as yet unidentified regulatory regions of *Smc6*. To be certain that our *jnj* alleles corresponded to *Smc6*, we generated additional *Smc6* lines by imprecise excision of the P-element present in line *NP2592*, including the new line jnj^{X1} that lacks exon 1 and sequences up- and downstream of this exon (Figure 2.4A). We tested caffeine sensitivity in all of the *jnj* allelic combinations and found that raising larvae on 0.5 mM caffeine resulted in almost complete lethality (Figure 2.3B). Using RNAi to deplete *Smc6* expression in developing eye discs also resulted in a caffeine-dependent rough eye phenotype (Figure 2.5B). Collectively, the presence of a frame shift mutation in *Smc6* in jnj^{R1}, the reduced expression levels of *Smc6* in all seven alleles of *jnj*, the caffeine-dependent lethality of the deletion allele jnj^{X1}, and caffeine-dependent eye phenotypes induced by *Smc6* RNAi all implicate *CG5524/Smc6* as the relevant gene in *jnj* mutants.

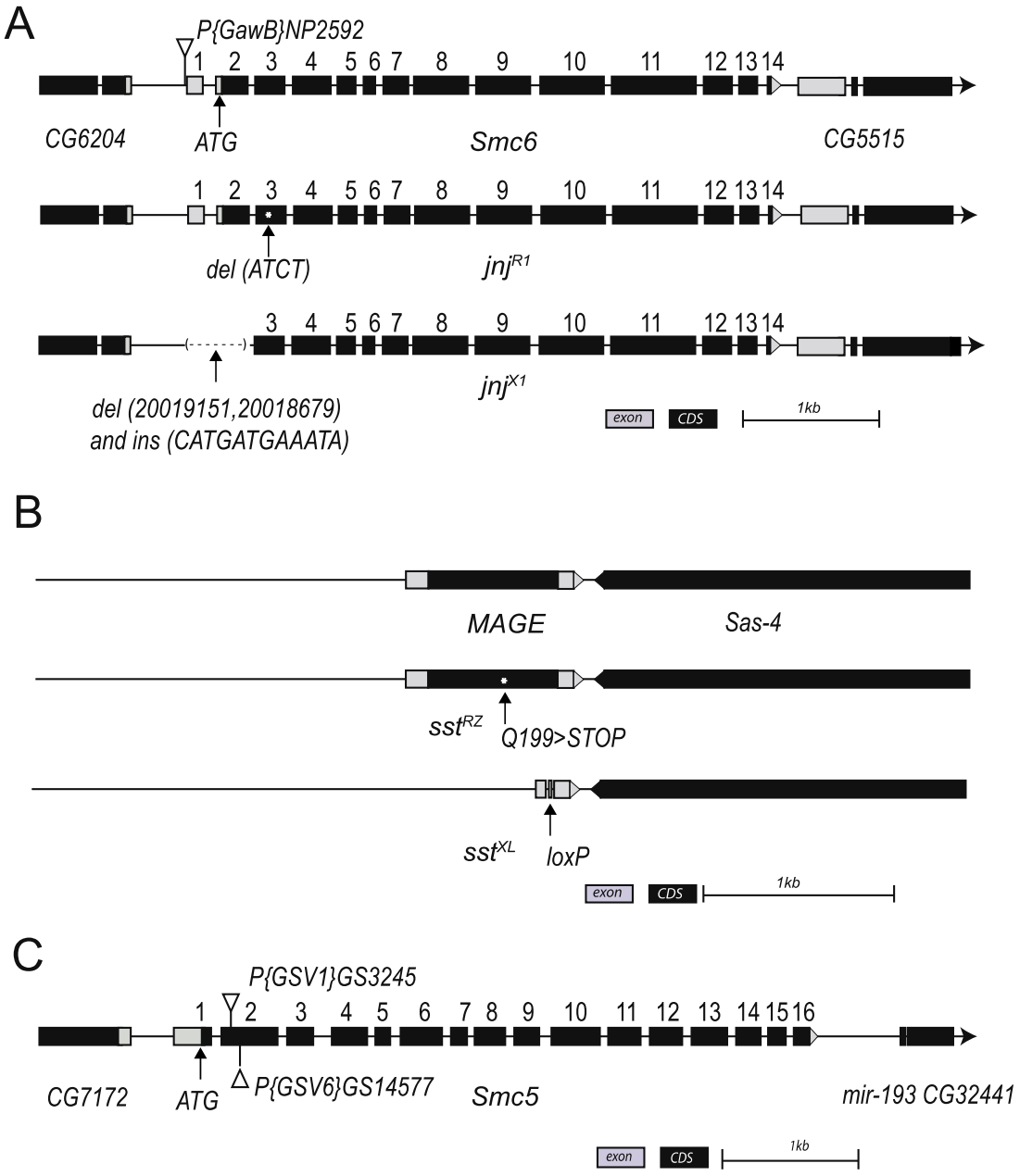


Figure 2.4. Overview of *Smc6, MAGE*, and *Smc5* gene location, structural organization and mutant alleles.
(A) *Smc6* is a 14 exon gene located on 3R:95E8-95F1. jnj^{R1} contains a 4 bp deletion in the 2nd coding exon. jnj^{X1} contains a 473 bp deletion of sequences upstream of exon 1 (196 bp), the entire exon 1 (252 bp), and a portion of intron 1 (25 bp), with a 12 bp vestige of the original P element remaining. *Smc6* genomic locus (3R:20,014,770..20,019,145 [-]) is shown. (B) *MAGE* is a single exon gene located on the right arm of the 3rd chromosome at position 84C7-84C7. sst^{RZ} has a point mutation that converts a glutamine at position 109 to a stop codon. sst^{XL} carries a targeted deletion of the entire coding sequence of *MAGE*. *MAGE* genomic locus (3R:2,979,960..2,980,898 [-]) is shown. (C) *Smc5* is a 16 exon gene located in 78D6-78D7 of the left arm of the 3rd chromosome. Exons encoding the longest transcripts are shown. Both *P{GSV1}GS3245* and *P{GSV6}GS14577* are inserted in the second coding exon. The *Smc5* genomic locus

(3L:21,562,309..21,566,623 [+]) is shown. CDS, coding sequence. (A,B) generated by RZ and XL, graphed by XL. (C) Ordered from Bloomington, confirmed and graphed by XL .

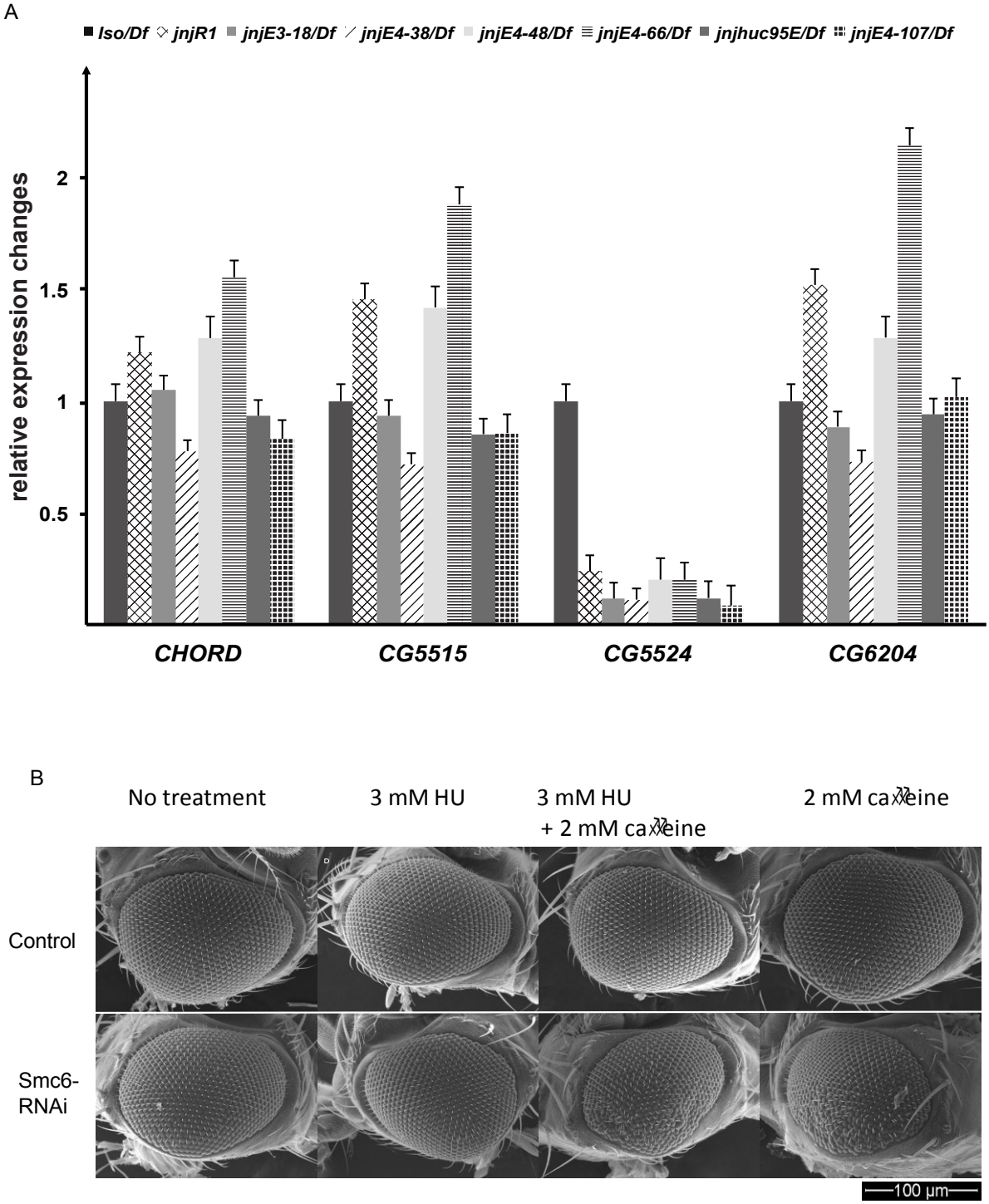


Figure 2.5. Caffeine sensitivity of *jnj* alleles is caused by loss of *Smc6*.
(A) mRNA transcript levels of *Smc6* and its neighboring genes *CHORD, CG5515* and *CG6204* in control and *jnj* mutant flies were measured by quantitative RT-PCR. All seven *jnj* alleles tested had reduced *Smc6* transcript levels ranging from 7% to 24% of the control level, while the

transcript levels of the neighboring genes comparable to the control level. The caffeine screen starting stock "Iso" carrying the transgenic *FRT82B* site crossed to Df to normalize the *Smc6* level was used to generate control flies. "Df" is the deficiency chromosome *Df(3R)Exel6198*. (B) Knocking-down *Smc6* expression using RNAi in developing eye discs resulted in a caffeine-dependent adult rough eye phenotype. Control, *Eyeless-Gal4/+* was from a cross of *Eyeless-Gal4/Eyeless-Gal4* X w^{1118} and *Smc6*-RNAi, *Eyeless-Gal4/+; UAS-Smc6-RNAi/+* resulted from the cross *Eyeless-Gal4/Eyeless-Gal4* X *UAS-Smc6-RNAi/+*. *UAS-Smc6-RNAi* was obtained from VDRC (#107055) (A) by RZ, (B) by XL.

2.3.3 Caffeine-sensitivity in *sleepless in seattle* mutants is due to mutations in the *MAGE* gene

The sst^{RZ} mutation exhibits caffeine-dependent pupal lethality in combination with a chromosomal deficiency (*Df(3R)Antp*^{1}, Figure 2.3C) but sst^{RZ} homozygotes are not viable on regular media, presumably because of a second site mutation. Further deletion mapping refined the position of the caffeine-sensitive *sst* locus to a region containing seven candidate genes, each of which were sequenced. We identified a glutamine to stop mutation affecting the *MAGE* gene (*61*) in sst^{RZ}, at position 109 of the 232 amino acid MAGE protein (Figure 2.4B). In previous studies, depletion of *MAGE* mRNA using double strand RNA injection suggested that *MAGE* was essential for viability during early embryogenesis, whereas conditional knockdown at later developmental stages suggested a role in postembryonic neuronal survival and proliferation (*62*). Moreover, DNA fibers connecting mitotic cells were observed after RNAi-mediated depletion of *Smc5* or *Smc6* in *S2* cells, suggesting that the Smc5/6 complex could be essential for mitosis in *Drosophila* (*47*). We therefore initially reasoned that sst^{RZ} was a partial loss-of-function allele, since hemizygous sst^{RZ} flies were viable. To test this idea we synthesized a knockout allele by homologous recombination (*54*). In this new allele (sst^{XL}) the complete coding sequence of *MAGE* was deleted (Figure 2.4B). Surprisingly, homozygous sst^{XL} flies displayed no increased lethality or obvious mutant phenotypes when raised

on media without caffeine. As with sst^{RZ} hemizygotes, sst^{XL} flies reared in caffeine media were inviable, but they were less sensitive to a lower dose of caffeine (0.5 mM) than *jnj* mutants (Figure 2.3C). About 15% of predicted sst^{XL} homozygous flies survived 2 mM caffeine exposure and the surviving flies often had small or rough eyes, similar to sst^{RZ} mutants (Figure 2.3A). Transheterozygous sst^{RZ}/sst^{XL} progeny were also viable on normal media, but only 6% survived on 2 mM caffeine (Figure 2.3C). Using polyclonal antibodies directed against MAGE (*55*) we found that MAGE was absent from protein lysates derived from *sst* adult flies (Figure 2.6). In addition, caffeine-dependent lethality of sst^{XL} can be complemented by a genomic *MAGE* transgene (Table 1.1) that includes the full coding region of *MAGE* and 3 kb sequence upstream and expresses MAGE protein at normal levels (Figure 2.6). Collectively, the identification of a stop mutation in the *MAGE* gene (sst^{RZ}), the caffeine-sensitivity of a *MAGE* knockout allele sst^{XL}, the loss of MAGE protein in *sst* flies and the rescue of caffeine sensitivity by a *MAGE* transgene all implicate *MAGE* as the mutated gene in *sst* flies.

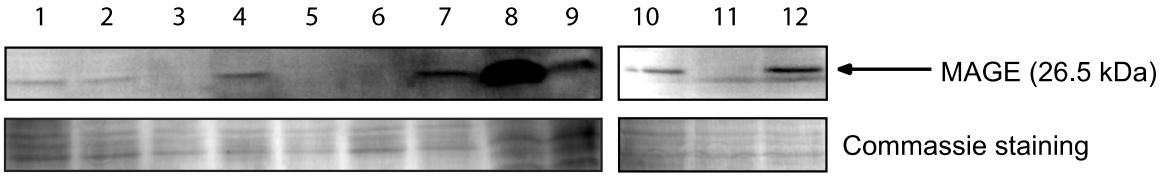


Figure 2.6. Immunoblot for MAGE.
Levels of endogenous MAGE were measured in protein lysates from whole flies derived from various lines, immunoblotted with anti-MAGE antibody. Genotypes were as follows: Lane 1: sst^{XL}*/TM3,Sb*, 2: sst^{RZ}*/TM3,Ser,ActGFP*, 3: sst^{XL}/sst^{RZ}, 4: *Df(3R)Antp1/TM3,Sb*, 5: *Df(3R)Antp1/*sst^{RZ}, 6: *Df(3R)Antp1/*sst^{XL}, 7. w^{1118}, 8: *S2* cells, 9: *S2* cells dMAGE RNAi, 10: sst^{XL}/TM3,Ser,ActGFP, 11: sst^{XL}/sst^{XL}, 12: *3Kb+MAGE transgene/CyO;* sst^{XL}/sst^{XL}. Done by XL.

2.3.4 Smc5 mutant flies are caffeine sensitive

In yeasts and mammalian cells, all known SMC6 functions involve SMC5 (*29, 63*), so we predicted that loss of *Smc5* activity would also cause caffeine sensitivity in flies (Figure 2.7A). We tested two P insertion alleles predicted to affect *Smc5* for caffeine sensitivity, namely $Smc5^{P\{GSV1\}GS3245}$, referred to as $Smc5^{P5}$, and $Smc5^{P\{GSV6\}GS14577}$, referred to as $Smc5^{P7}$ (*64*). As predicted, both *Smc5* mutants were sensitive to caffeine (Figure 2.3D). Both of these alleles have P-element insertions within the second exon of *Smc5* and the insertion sites are very close to the putative start codon (Figure 2.4C). Therefore, they are very likely to be null alleles. To rule out the possibility that caffeine-sensitivity of *Smc5* flies was caused by second site mutations, we generated fly lines in which the P-elements in both alleles were excised by a transposase, either restoring the wild-type sequence or resulting in an insertion or deletion of the original P element insertion in the coding exon of *Smc5*. We therefore predicted that some excision lines would no longer be caffeine-sensitive while others would retain the mutant phenotype. As expected, of 13 independent fly lines produced by the excision of *P7*, seven lines were no longer caffeine sensitive (Table 2.2A). Similar results were obtained from the excision of *P5* (Table 2.2B). In conclusion, as with *Smc6* and *MAGE*, loss of *Smc5* function results in caffeine-dependent lethality.

2.3.5 Caffeine sensitivity is mediated through Smc5/6

At the whole organism level, a higher proportion of *MAGE* mutants were able to survive exposure to 0.5 mM caffeine throughout larval development than *Smc6* and *Smc5* mutants. Indeed all genetic combinations of *MAGE* mutant flies

had some survivors on media containing 2 mM caffeine, while there were essentially no survivors among the *Smc5* or *Smc6* mutants raised on 2 mM caffeine (Figure 2.3B-D). This suggests that the MAGE protein is less important for caffeine resistance than the Smc5 and Smc6 proteins. To further test this hypothesis, we measured the viability of flies carrying mutations in two different components of the protein complex when raised on media containing caffeine. Flies deficient for both MAGE and Smc6 were more sensitive to caffeine than flies deficient for MAGE alone, but were similar in sensitivity to flies deficient for Smc6 alone (Table 2.3). This suggests that the Smc5/6 heterodimer has a more critical role in caffeine resistance than does the sub-complex containing Nse1-MAGE, consistent with observations in yeasts (*1*).

2.3.6 *Drosophila* Smc5/6 components form a protein complex

In yeasts, the Smc5/6 complex consists of Smc5, Smc6 and six Nse (non-Smc element) subunits (*65*), four of which were also identified in humans (*29, 40*). In searches of *Drosophila* genome databases, we uncovered a set of putative transcription units that appear to correspond to SMC5/6 complex subunits in yeasts (Table 2.4). Of these, *MAGE* has previously been described as a homolog of yeast *Nse3* and human *MAGEG1* (*29*). In *Drosophila*, MAGE protein was shown to interact with *Drosophila* Nse4 (Nse4) using a yeast two-hybrid system (*66*). When we examined the Gene Expression Omnibus (GEO, (*67*)) to compare gene expression profiles, we found that these two genes have very similar expression patterns across different tissues, supporting the idea that the encoded proteins function in a complex. Yeast Nse1 has been detected in the same sub-

complex as Nse3 and Nse4, as part of the larger Smc5/6 complex (Figure 2.7A) (*65*). We first tested for a physical interaction between *Drosophila* MAGE and Nse4 in cell culture, by generating epitope-tagged plasmid constructs that produce HA-tagged Nse4 or FLAG-tagged MAGE, and co-transfecting them into *Drosophila* Schneider 2 (S2) cells. We were able to co-immunoprecipitate HA-Nse4 and FLAG-MAGE from S2 cell lysates (Figure 2.7B). We then performed *in vitro* pull down experiments to show that this interaction is likely direct, and that MAGE also interacts with Nse1 directly (Figure 2.7C). These results indicate that the three *Drosophila* proteins (Nse1, MAGE and Nse4) form a sub-complex analogous to that found in yeast, consistent with conservation of structure across species.

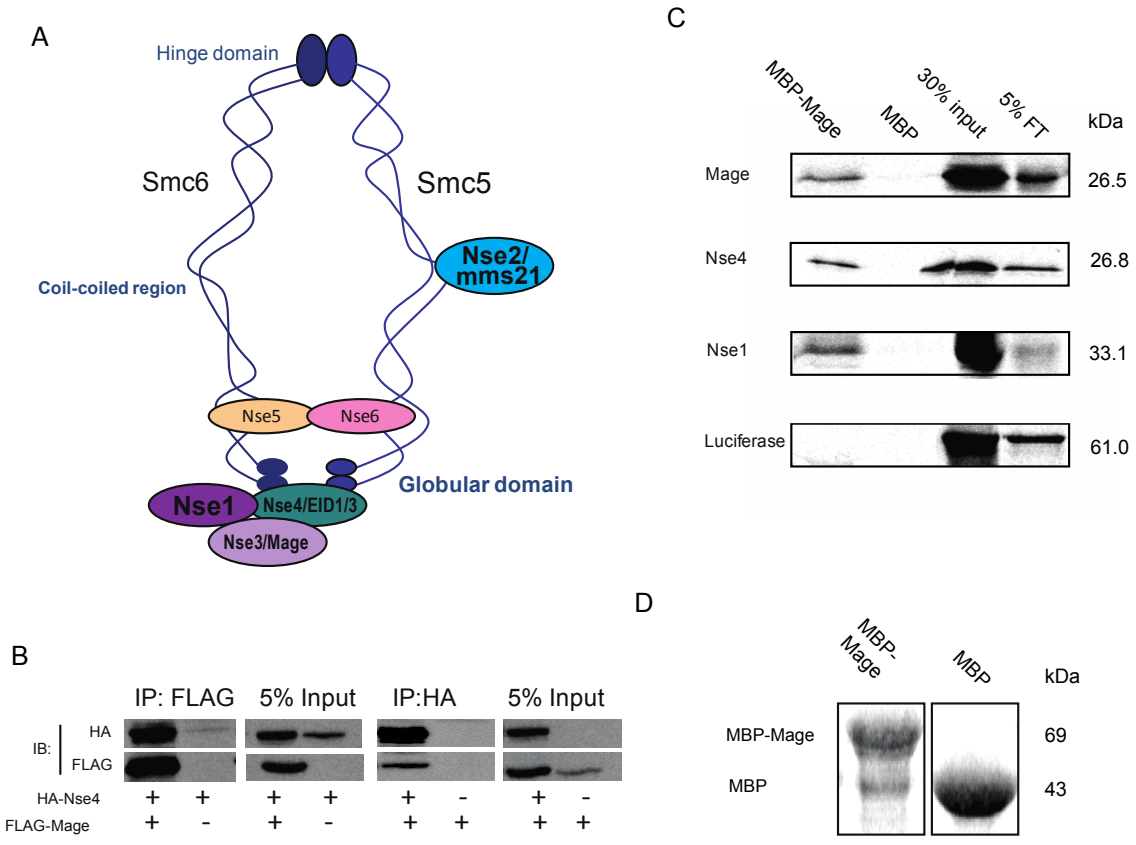


Figure 2.7. MAGE is part of the *Drosophila* Smc5/6 complex.
(A) Diagram of a generic Smc5/6 complex in *S. pombe* (adapted from (*68*)). The structure in *S. cerevisiae* is different in that Nse5/6 were found to bind at the hinge. (B) MAGE interacts with Nse4 when both proteins are co-expressed in *S2* cells. HA-Nse4 co-immunoprecipitated (co-IP) with FLAG-MAGE from an *S2* cell lysate when two proteins were co-expressed; FLAG-MAGE co-IPed with HA-Nse4 from the *S2* cell lysate when two proteins were co-expressed. (C) Recombinant MAGE interacts with Nse4 and Nse1 directly. Immobilized maltose binding protein (MBP)-fused MAGE or MBP were incubated with ^{35}S-methionine labeled MAGE, Nse4, Nse1, or luciferase (as a negative control), respectively. Proteins that were associated with immobilized MBP-MAGE or MBP were resolved with SDS-PAGE and visualized by autoradiography. Results show that MAGE, Nse4, and Nse1 each interact with MBP-MAGE but not with MBP and luciferase does not interact with either of these proteins. (D) Coomassie staining of protein immobilized on 10 µl of amylose beads showed that approximately equal amounts of MBP-MAGE and MBP proteins were immobilized on resin beads. Done by XL.

2.3.7 Loss of function for *Smc6* or *MAGE* sensitizes imaginal cells to caffeine-induced apoptosis

Previous examination of jnj^{huc95E} hemizygous mutants were based on the EGUF eye mosaic system (*52*). We observed caffeine-dependent defects in ommatidial patterning and increased apoptosis in the eye discs. Larvae mutant for *Smc6* or *MAGE* die at the pupal stage when raised on caffeine-containing media. Remarkably, upon dissection of these larvae we noticed that the imaginal discs were severely damaged or altogether absent, suggesting increased cell death as the cause of this defect. To test this idea, we dissected eye imaginal discs from late third instar larvae and labeled them with antibodies against activated caspase 3 to mark apoptotic cells. We detected minimal labeling of apoptotic foci in eye discs of control larvae, regardless of caffeine exposure (Figure 2.8). In contrast, dramatically increased labeling of apoptotic foci were seen in the eye discs of *Smc6* or *MAGE* mutant third instar larvae after 12 hours of caffeine exposure. Apoptotic labeling was markedly enhanced in a band of cells immediately anterior to the morphogenetic furrow, where cells become synchronized in G1 phase (*69*).

These results suggest that caffeine-induced apoptosis in developing imaginal discs likely underlies caffeine-dependent pupal lethality in *MAGE* and *Smc6* mutant flies.

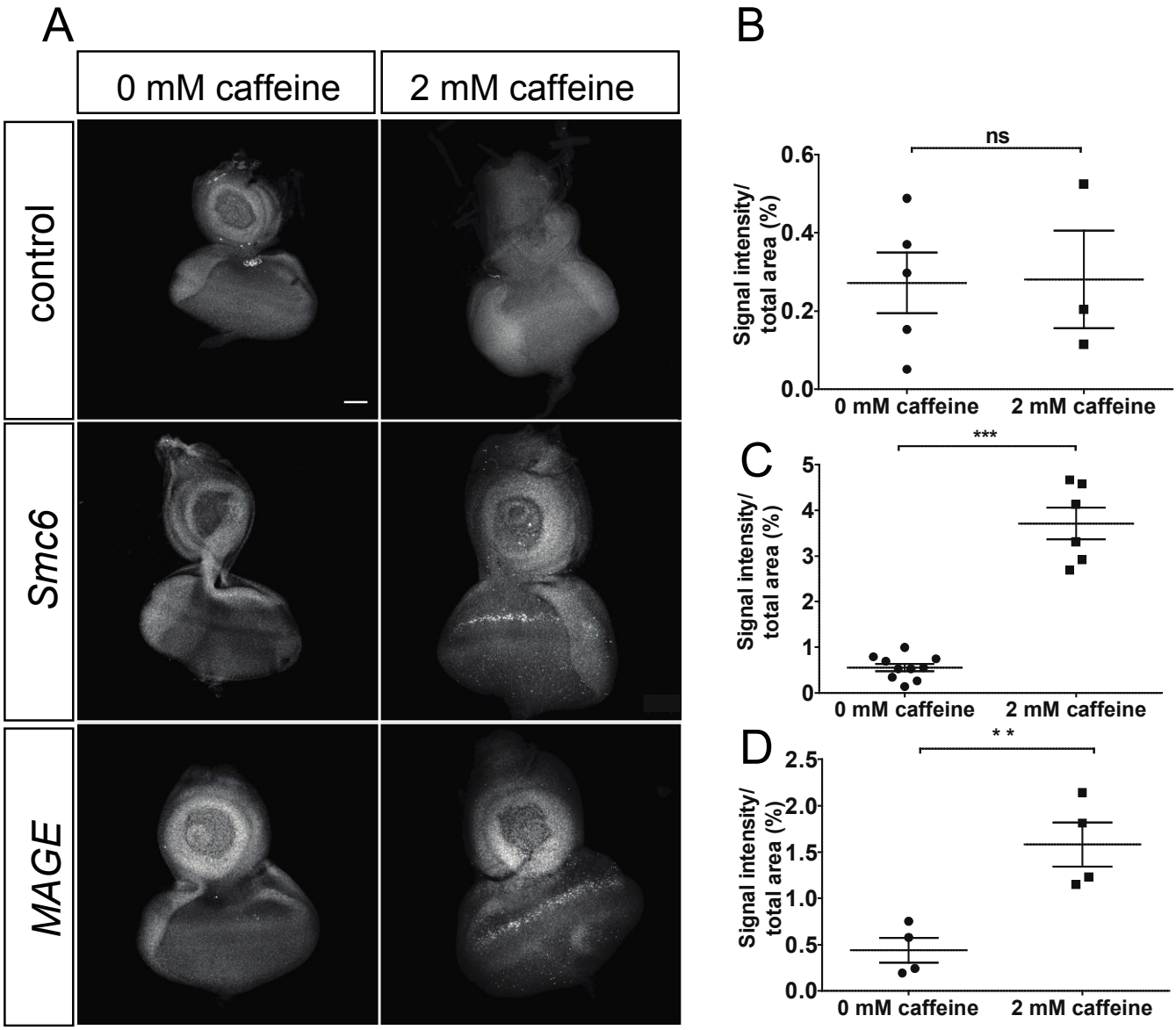
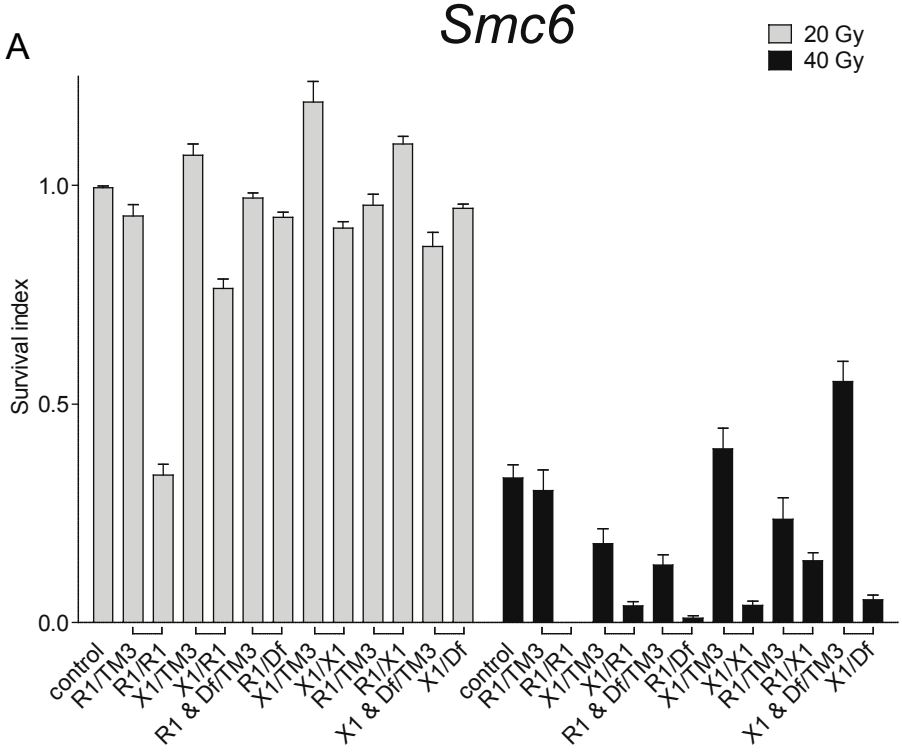


Figure 2.8. Caffeine exposure results in apoptosis in eye discs of MAGE and Smc6 mutants. (A) Anti-cleaved-caspase-3 antibody staining of eye discs from third instar larvae of control (WT, FRT82B), *MAGE* (sst^{RZ}/sst^{XL}), and *Smc6* (jnj^{X1}/jnj^{R1}) genotypes raised in either regular media (0 mM caffeine) or media supplemented with 2 mM caffeine for 12 hours before dissection. IMAGEs are single stacks of confocal iMAGEs. More cleaved-caspase-3 foci in eye discs of sst^{RZ}/sst^{XL} and jnj^{X1}/jnj^{R1} larvae were observed after caffeine exposure. A narrow band of apoptotic cells anterior to the presumptive morphogenetic furrow are most noticeable. Scale bar represents 50 μM. (B-D) Quantification and comparison of cleaved caspase-3 staining levels in WT (B),

MAGE (C) or Smc6 (D) eye discs, comparing the no caffeine and 2 mM caffeine groups. Data represent mean area stained from multiple eye discs for each genotype per treatment. A maximum projection of all stacks of a confocal iMAGE was used to quantify the signal intensity of staining. This value was divided by the area of each eye disc to obtain a ratio representing the relative amount of immunostaining. Error bars represent SEM. A non-paired two-tailed t-test was used to determine statistical significance. **, P=0.006, ***, P<0.0001. Done by RX, quantified by XL.

2.3.8 *Smc5/6* mutant flies are hypersensitive to genotoxic stress

The DNA damage response is a multi-step process that involves sensing of damage, cell cycle arrest, and repair of the damaged DNA. Yeast with hypomorphic mutations affecting *Smc6*, *Nse1*, *Nse2*, *Nse3* or *Nse4* are hypersensitive to gamma irradiation, UV light, MMS, camptothecin (a topoisomerase I inhibitor), and inhibition of DNA replication by HU (*16, 25, 63, 70, 71*). All of these genotoxic stresses directly or indirectly generate DNA single-stranded or double-stranded breaks. To explore whether *Drosophila* Smc5/6 provides similar responses to genotoxic stress, we analyzed the effects of ionizing radiation, camptothecin, HU or MMS on viability. Exposure to 40 Gy ionizing radiation caused increased lethality in *MAGE*, *Smc6* and *Smc5* mutants compared to controls (Figure 2.9). Moreover, all three mutants were hypersensitive to camptothecin, HU and MMS, compared to controls (Figure 2.10).


A
Smc6
20 Gy
40 Gy
Survival index
1.0
0.5
0.0
control
R1/TM3
R1/R1
X1/TM3
X1/R1
R1 & Df/TM3
R1/Df
X1/TM3
X1/X1
R1/TM3
R1/X1
X1 & Df/TM3
X1/Df
control
R1/TM3
R1/R1
X1/TM3
X1/R1
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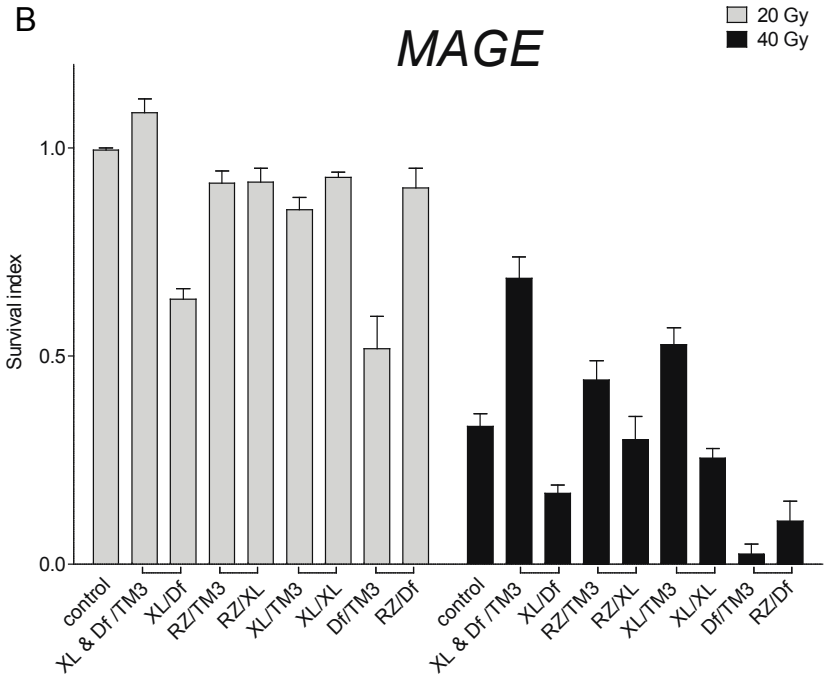
R1/X1
X1 & Df/TM3
X1/Df


B
MAGE
20 Gy
40 Gy
Survival index
1.0
0.5
0.0
control
XL & Df /TM3
XL/Df
RZ/TM3
RZ/XL
XL/TM3
XL/XL
Df/TM3
RZ/Df
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XL/XL
Df/TM3
RZ/Df

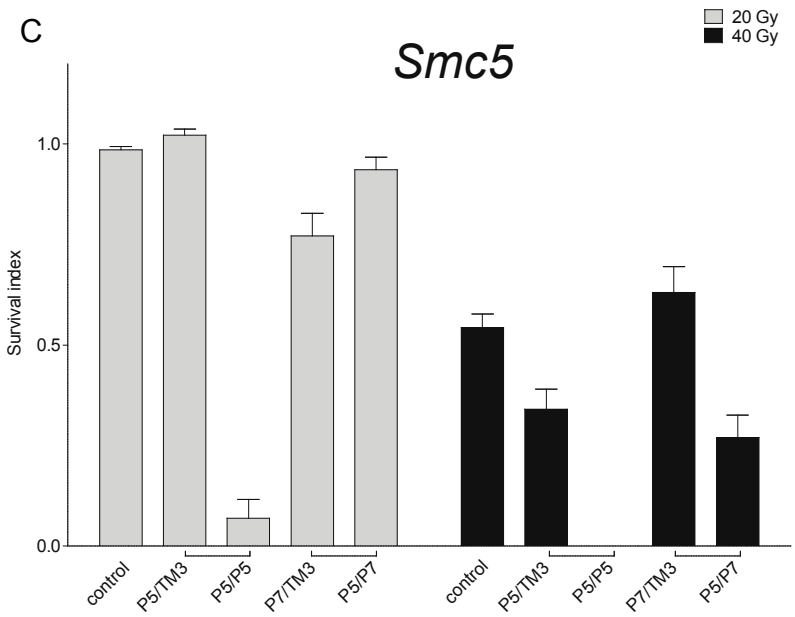

Figure 2.9. Smc5/6 mutants are hypersensitive to ionizing radiation.
(A-C) *Smc6*, *MAGE* or *Smc5* homozygous, trans-heterozygous or hemizygous mutants have reduced survival when exposed to 40 Gy of IR. Bars represent the survival index (p) ± SEM. "␣" indicates flies eclosed from the same cross. Absence of a bar indicates that no flies survived at that IR dose. (A) Smc6 mutants are hypersensitive to IR. R1 (jnj^{R1}) and X1 (jnj^{X1}) are *Smc6* alleles. Df *(Df(3R)Exel6198)* is a deficiency chromosome uncovering the *Smc6* locus. (B) MAGE mutants are hypersensitive to IR. RZ (sst^{RZ}) and XL (sst^{XL}) are *MAGE* alleles. *Df (Df(3R)Antp1)* is a deficiency chromosome uncovering the MAGE locus. (C) Smc5 mutants are hypersensitive to IR. *P5 (Smc5P{GSV1}GS3245)* and *P7 (Smc5P{GSV6}GS14577)* are *Smc5* alleles. *Df (Df(3L)BSC418)* is a deficiency chromosome uncovering the *Smc5* locus.
Done by XL.

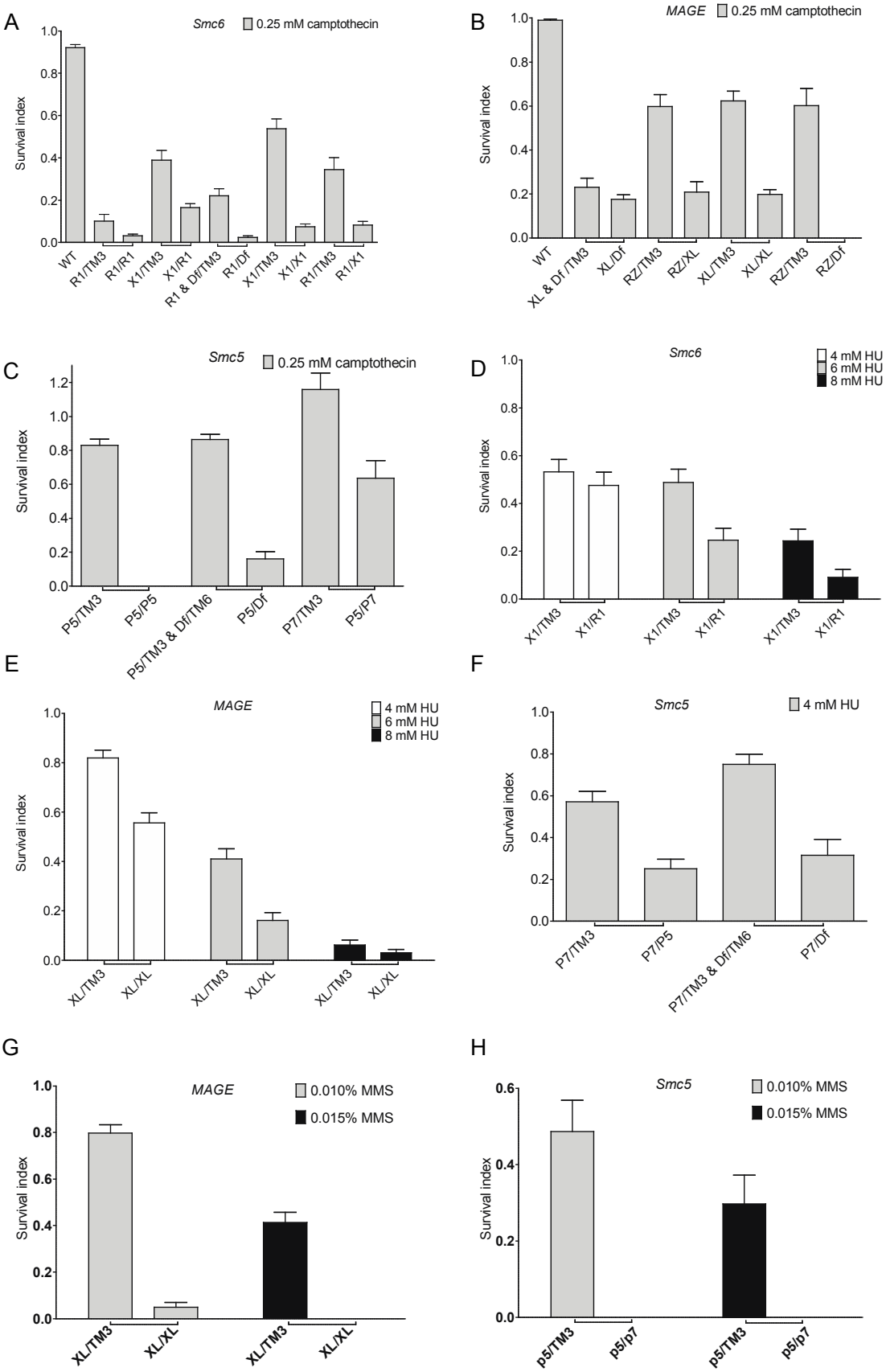
A
Smc6
0.25 mM camptothecin
Survival index
WT
R1/TM3
R1/R1
X1/TM3
X1/R1
R1 & Df/TM3
R1/Df
X1/TM3
X1/X1
R1/TM3
R1/X1
B
MAGE
0.25 mM camptothecin
Survival index
WT
XL & Df /TM3
XL/Df
RZ/TM3
RZ/XL
XL/TM3
XL/XL
RZ/TM3
RZ/Df
C
Smc5
0.25 mM camptothecin
Survival index
P5/TM3
P5/P5
P5/TM3 & Df/TM6
P5/Df
P7/TM3
P5/P7
D
Smc6
4 mM HU
6 mM HU
8 mM HU
Survival index
X1/TM3
X1/R1
X1/TM3
X1/R1
X1/TM3
X1/R1
E
MAGE
4 mM HU
6 mM HU
8 mM HU
Survival index
XL/TM3
XL/XL
XL/TM3
XL/XL
XL/TM3
XL/XL
F
Smc5
4 mM HU
Survival index
P7/TM3
P7/P5
P7/TM3 & Df/TM6
P7/Df
G
MAGE
0.010% MMS
0.015% MMS
Survival index
XL/TM3
XL/XL
XL/TM3
XL/XL
H
Smc5
0.010% MMS
0.015% MMS
Survival index
p5/TM3
p5/p7
p5/TM3
p5/p7

Figure 2.10. *Smc6*, *MAGE* and *Smc5* mutants are sensitive to camptothecin, HU and MMS. Flies eclosed from the same cross are indicated with a '␣' . Embryos (n=360, expected to be half homozygous or transheterozygous mutants and half heterozygous mutants) were collected from a given cross for each drug concentration and allowed to develop in media without or with each drug. Bars represent the survival index (p) ± SEM. Absence of a bar indicates that no flies survived at that drug concentration. The survival index was calculated by normalizing the number of eclosed adults from each drug treatment against the number of eclosed adults from the no treatment control. (A-C) *Smc6*, *MAGE* or *Smc5* homozygous, trans-heterozygous or hemizygous mutants have reduced survival when raised in media supplemented with 0.025 mM camptothecin; (D-F) *Smc6*, *MAGE* or *Smc5* homozygous, trans-heterozygous or hemizygous mutants have reduced survival when raised in media supplemented with hydroxyurea (HU); (G) *MAGE* mutants are sensitive to MMS; (H) *Smc5* mutants are sensitive to MMS. *Smc6* mutants are also sensitive to MMS (data not shown). *Smc6*: R1 (jnj^{R1}) and X1 (jnj^{X1}) are *Smc6* alleles. *Df (Df(3R)Exel6198)* is a deficiency chromosome uncovering the *Smc6* locus; *MAGE*: RZ (sst^{RZ}) and XL (sst^{XL}) are *MAGE* alleles. *Df (Df(3R)Antp1)* is a deficiency chromosome uncovering the *MAGE* locus. *Smc5*: *P5 (Smc5P{GSV1}GS3245)* and *P7 (Smc5P{GSV6}GS14577)* are *Smc5* alleles. *Df (Df(3L)BSC418)* is a deficiency chromosome uncovering the Smc5 locus. Done by XL.

2.3.9 Loss of *Smc5/6* function does not compromise G2/M and S phase checkpoints induced by genotoxic agents

Studies in *Drosophila* have proven to be valuable for the study of proteins and pathways controlling DNA repair and checkpoint responses, which are remarkably well conserved among flies and other organisms (*47, 72*). In *S. cerevisiae*, *nse3-1* hypomorphic mutants activate a DNA damage checkpoint that arrests cells in late S phase/G2 (*16*), and in *S. pombe*, *Smc6 (Rad18)* is required for maintenance but not activation of the G2 checkpoint (*26*). We therefore tested whether cell cycle checkpoints important for DNA damage response pathways were perturbed in caffeine-sensitive *MAGE* or *Smc6* mutant flies. To assess G2/M checkpoint function we used ionizing radiation (IR) to determine if IR exposure decreased the number of mitotic cells (*73*). We dissected eye imaginal discs from late third instar larvae and labeled them with anti-phospho histone H3 antibodies to mark mitotic cells. The number of mitotic cells in un-irradiated eye imaginal discs of jnj^{R1} *(Smc6)* or sst^{XL} *(MAGE)* larvae was comparable to that of control

eye discs (Figure 2.11A, *MAGE* not shown for simplicity). Larvae were exposed to 40 Gy of IR and dissected eye discs were examined from 15 to 120 min. after exposure. Phospho-histone H3 foci disappeared after 30 or 60 min in wild-type (*Iso)* controls, $jnj^{R1/X1}$ *(Smc6)* and $sst^{XL/RZ}$ *(MAGE)* eye discs (Figure 2.11A), demonstrating that neither MAGE nor Smc6 is required for activation of the G2/M checkpoint.

The caffeine sensitive ATM/ATR kinases are important mediators of DNA damage checkpoints (*48*). In *S. pombe*, the SMC5/6 complex is recruited to and stabilizes stalled replication forks after Rad3 (ATR homolog) activation (*74*). To investigate whether the S phase checkpoint was intact in $jnj^{R1/X1}$ *(Smc6)* and $sst^{XL/RZ}$ *(MAGE)* mutant flies, we monitored BrdU incorporation pattern in eye imaginal discs before and after treatment with HU, which induces the S phase checkpoint. We observed many S-phase cells incorporating BrdU in control untreated eye discs, however incorporation was abolished upon exposure to HU. BrdU incorporation was also abolished by HU treatment in $jnj^{R1/X1}$ and $sst^{XL/RZ}$ mutant discs (Figure 2.11B), demonstrating that MAGE and Smc6 are also not essential for S phase checkpoint activity in *Drosophila*.

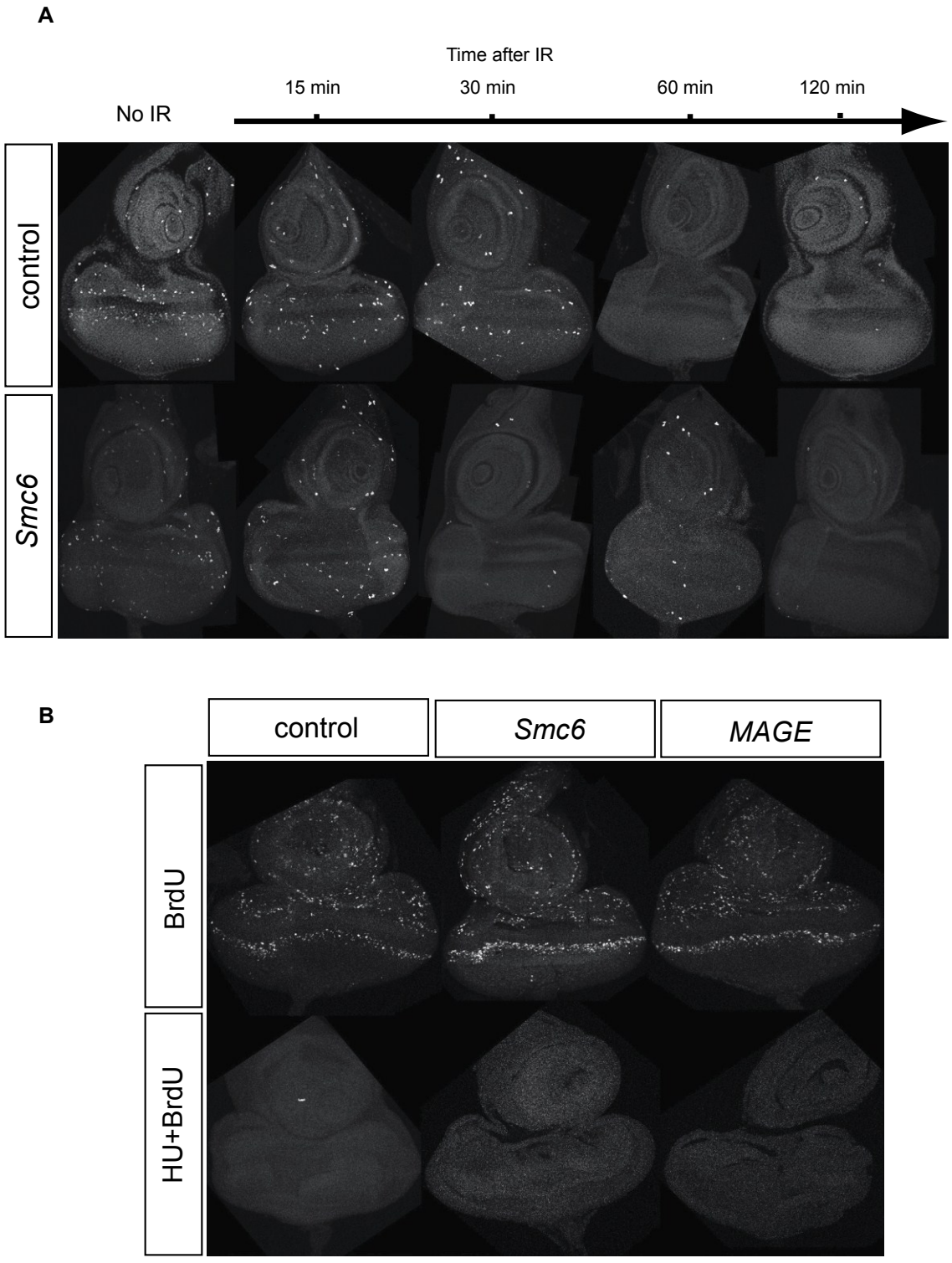

Figure 2.11. Smc5/6 genes are not required for G2/M and S phase checkpoints induced by genotoxic agents.
(A) Wandering third instar larvae were irradiated with 40 Gy of ionizing radiation and the eye-antenna discs were dissected and fixed 15 minutes, 30 minutes, 1 hour or two hours after radiation, with discs from unirradiated larvae serving as controls. Representative iMAGEs of PH3 staining

for mitotic cells in eye-antenna discs from control (WT, FRT82B) and *Smc6*, (jnj^{R1}/jnj^{X1}) transheterozygous larvae are shown. (B) Eye-antenna discs from wandering third instar larvae were incubated with or with out HU before adding BrdU to the incubation solution. Representative iMAGEs of BrdU staining for cells in S phase in eye-antenna discs from control (WT, FRT82B), transheterozygous *Smc6* (jnj^{R1}/jnj^{X1}) or transheterozygous *MAGE* (sst^{RZ}/sst^{XL}) eye-antenna discs are shown. Done by RZ.

2.3.10 *Smc6* and *MAGE* genetically interact with genes required for DNA damage responses

Caffeine inhibits ATR and ATM kinase activity (*49, 75*), raising the possibility that partial loss of ATM or ATR function could be contributing to the caffeine-induced defects that we observed in *Smc5/6* mutant flies. We therefore examined whether genetically reducing ATM or ATR function in an *Smc6* mutant background would cause synthetic lethality. The *Drosophila* homolog of ATR is Mei-41 (*56*) and *mei-41* mutants are homozygous viable but not caffeine-sensitive on their own (*52*). To test for genetic interactions between *mei-41* and *Smc6*, we generated double mutants and measured the proportion that survived to adulthood when raised on caffeine-free media. There was no increased lethality associated with *mei-41; Smc6* double mutants (Table 2.5), implying that the inhibition of ATR alone by caffeine was not the main cause of caffeine-dependent lethality of *Smc6* homozygotes. To further examine genetic interactions between *ATR* and *MAGE* or *Smc6*, we used the EGUF system as a more sensitive system for detecting mutant phenotypes than lethality. Raised on standard media, adult flies with homozygous *MAGE* mutant eyes were indistinguishable from control flies (Figure 2.12). Raised on 2 mM caffeine, however, *MAGE* mutant eyes were moderately rough relative to control eyes. *ATR* RNAi alone caused no observable roughness in the eye but when *ATR* RNAi was expressed in MAGE-deficient eyes

moderate to severely rough caffeine-dependent eye defects were observed that were not seen on caffeine-free media (Figure 2.12, quantification in Figure 2.13). We then tested whether ATM plays a role in caffeine sensitivity. *Drosophila ATM* (*tefu*) null mutants are non-conditional pupal lethal (*76*), so we used the *EGUF* system to examine these interactions as well. *ATM*-RNAi knockdown alone produced a normal looking eye, either in the absence or presence of caffeine. When *MAGE* mutant eyes were combined with *ATM*-RNAi, however, we observed a range of caffeine-dependent rough eye phenotypes, similar to eye defects caused by *ATR*-RNAi in *MAGE*-deficient eyes (Figure 2.12, 2.13). As for *ATM*-RNAi, *ATR*-RNAi knockdown alone produced a normal looking eye, either in the absence or presence of caffeine. We noted differences in expressivity between the *MAGE*-deficient eyes (compare Figs. 2.3A and 2.12A) that could be caused by slight differences in the genetic background (the genetic interaction study used *CyO* balancers while the original screen had wild type chromosomes) or the accumulation of genetic modifiers. We propose that the caffeine-induced partial loss of function of both ATM and ATR causes the rough eye phenotype in the *MAGE*-deficient background, and that further loss of either ATM or ATR increases the severity of this phenotype. We also examined interactions with *NBS1*, a component of the MRN (Mre11, Rad50 and Nbs1) complex that collaborates with ATM in DNA repair and telomere maintenance (*77*). While *NBS1*-knockdown alone produced no effect, a dramatic caffeine-dependent enhancement of the rough eye phenotype was observed when *NBS1*-RNAi was combined with eye-specific *MAGE* mutants (Figure 2.14). These striking caffeine-

dependent genetic interactions between *MAGE* and *ATR, ATM,* and *NBS1* suggest that these proteins act together in maintaining genome stability. Similar genetic interactions were observed between *ATR* and *ATM* in *Smc6* eye-specific mutants, supporting this conclusion.

2.3.11 *Drosophila MAGE RNAi* caffeine sensitive phenotype is rescued by Rad51 knockdown

In *Drosophila* and other organisms, Smc5/6 functions in the homologous recombination repair pathway in DNA double strand break repair (*19, 23, 24, 50*). Rad51 is a key component of the homologous recombination pathway, regulating the rate-limiting step of homology searching and strand invasion. In *Drosophila,* Smc5/6 prevents precocious Rad51 loading onto the irradiation damaged heterochromatin region before it moves outside of the HP1a domain for proper repair (*47*). In yeast, *Smc5/6* mutants accumulate unresolved DNA structures, and Smc5/6 actively resolves DNA mediated sister chromatin linkages (*78-80*). We therefore tested whether the caffeine-dependent rough eye phenotype of *Smc5/6* mutants is related to deregulated Rad51 activity. Knocking down *Rad51* in the *MAGE*-RNAi background rescued the rough eye phenotype of *MAGE*-RNAi flies in 80% of the double RNAi flies raised on 2 mM caffeine (Figs. 2.12B, 2.15). Taken together, these data indicate that the caffeine sensitivity of the Smc5/6 complex or at least of *MAGE* mutants is largely attributable to improper Rad51 activity.

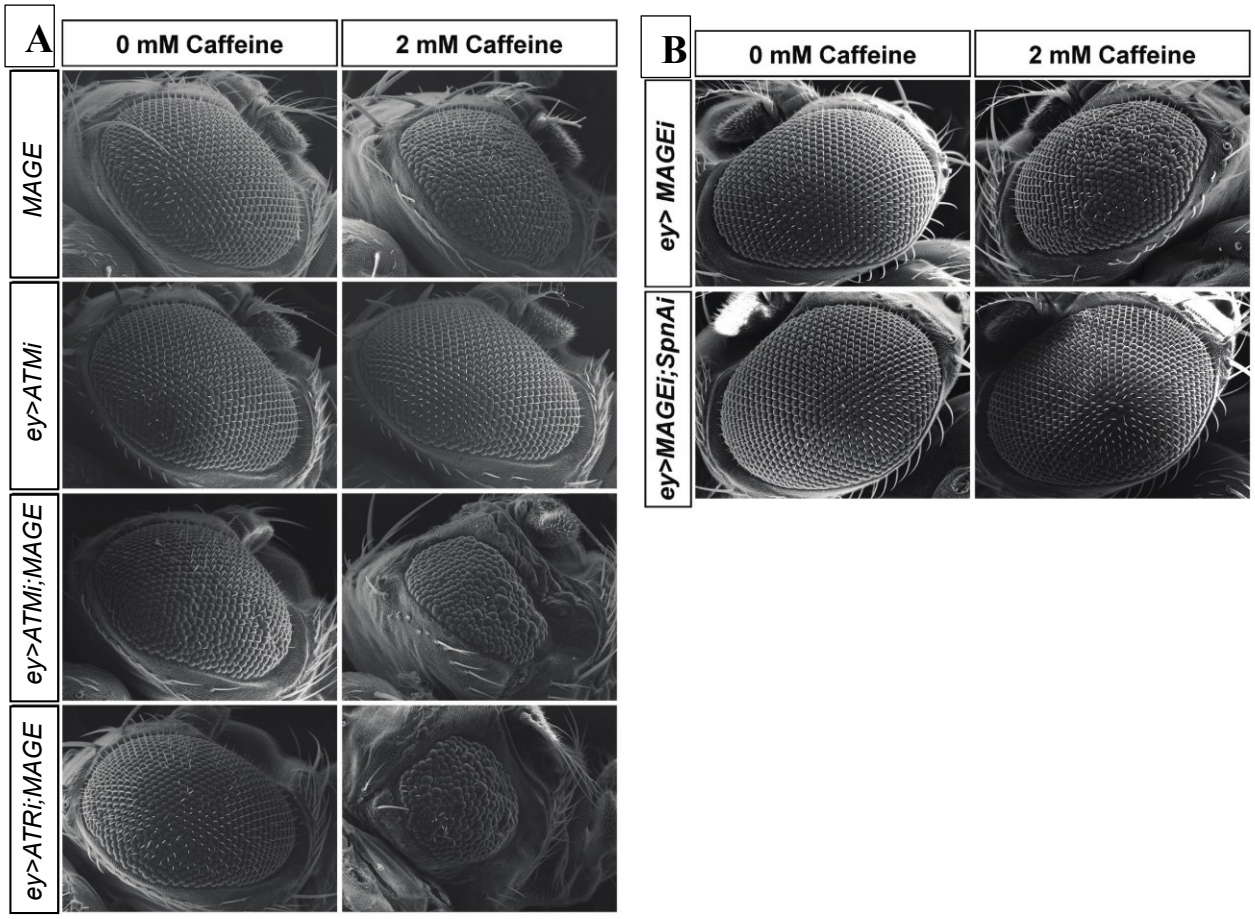

Figure 2.12. Caffeine-dependent genetic interaction of MAGE with ATM, ATR and Rad51(SpnA).

(A) Representative eye phenotypes of *MAGE (EGUF/+; FRT82B sstRZ/FRT82B GMR-hid*, loss of *MAGE* in eye cells), *ey>ATMi* (knockdown of *ATM* in eye cells), ey>ATMi;MAGE *(EGUF/UAS-ATM-RNAi;FRT82B sstRZ/FRT82B GMR-hid*, loss of MAGE and knockdown of ATM in eye cells) and *ey>ATRi;MAGE* (*EGUF/UAS-ATR-RNAi;FRT82B* sst^{RZ}*/FRT82B GMR-hid, loss of MAGE* and knockdown of *i* in eye cells) flies that were reared on either standard media or media containing 2 mM caffeine. The EGUF system carrying the eyeless-Gal4 driver was used to drive the UAS-RNAi transgenes in the eye and also makes the eye homozygous for *MAGE* (sst^{RZ}). Controls for the effects of each eyeless-driven RNAi alone were carried out for *ATM* and *ATR* resulting in wild type appearing eyes, but only the results of ATM RNAi are shown here as an example. (B) Representative eye phenotypes of MAGE knockdown (*eyeless-Gal4/+;UAS-MAGE-RNAi/UAS-Dicer2, knockdown of MAGE in eye cells*) and *MAGE Rad51* double knockdown (*eyeless-Gal4/UAS-SpnA-RNAi;UAS-MAGE-RNAi/UAS-Dicer2*, knockdown of *MAGE* and *Rad51* in eye cells) flies that were reared on either standard media or media containing 2 mM caffeine. Done by RZ.

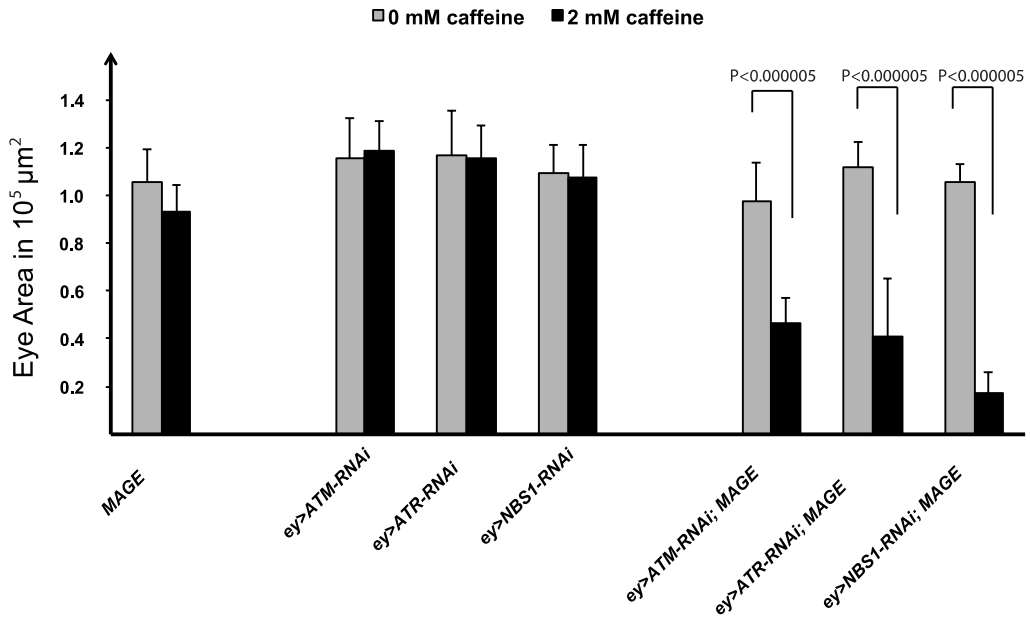

Figure 2.13. Quantification the area of the adult eye as a measure of the genetic interaction of *MAGE* with *ATM*, *ATR* or *NBS1*.
MAGE (EGUF/+; FRT82B sstRZ/FRT82B GMR-hid, loss of MAGE in eye cells), ey>ATM-RNAi (knockdown of ATM in eye cells), ey>ATR-RNAi (knockdown of ATR in eye cells), ey>NBS1-RNAi (knockdown of NBS1 in eye cells), ey>ATM-RNAi;MAGE (EGUF/UAS-ATM-RNAi;FRT82B sstRZ /FRT82B GMR-hid, loss of MAGE and knockdown of ATM in eye cells), ey>ATR-RNAi;MAGE (EGUF/UAS-ATR-RNAi;FRT82B sstRZ /FRT82B GMR-hid, loss of MAGE and knockdown of ATR in eye cells), and ey>NBS1-RNAi;MAGE (EGUF/UAS-NBS1-RNAi;FRT82B sstRZ/FRT82B GMR-hid, loss of MAGE and knockdown of NBS1 in eye cells) flies were reared on either standard media or media containing 2 mM caffeine. A Student two-tailed t-test was performed to compare between genotypes. Done by RZ.

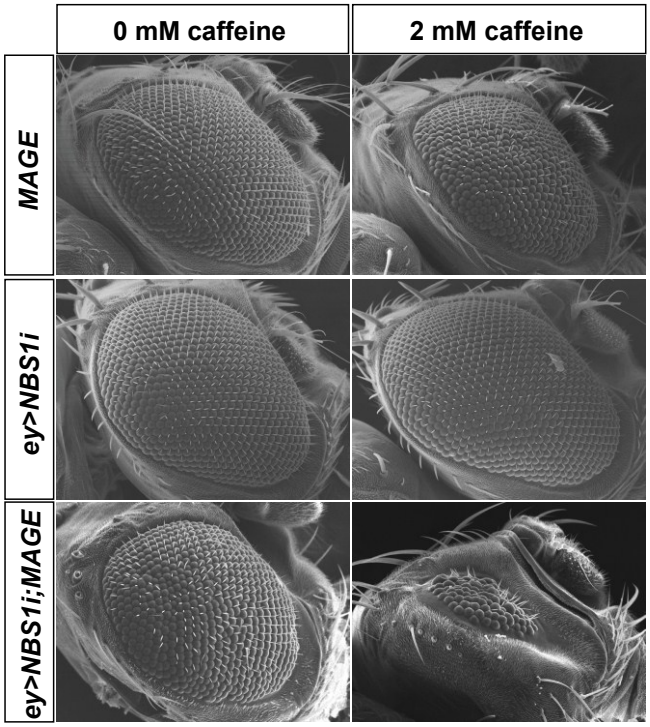

Figure 2.14. NBS1 interacts with MAGE.
Representative eye phenotypes of MAGE (EGUF/+; FRT82B sstRZ/FRT82B GMR-hid, loss of MAGE in eye cells) and ey>NBS1i (knockdown of NBS1 in eye cells) and ey>NBS1i;MAGE (EGUF/UAS-NBS1-RNAi;FRT82B sstRZ/FRT82B GMR-hid, loss of MAGE and knockdown of NBS1 in eye cells) flies that were reared on either standard media or media containing 2 mM caffeine. The EGUF system carrying the eyeless-Gal4 driver was used to drive the UAS-RNAi transgene in the eye and was also made the eyes homozygous for sstRZ. Done by RZ.

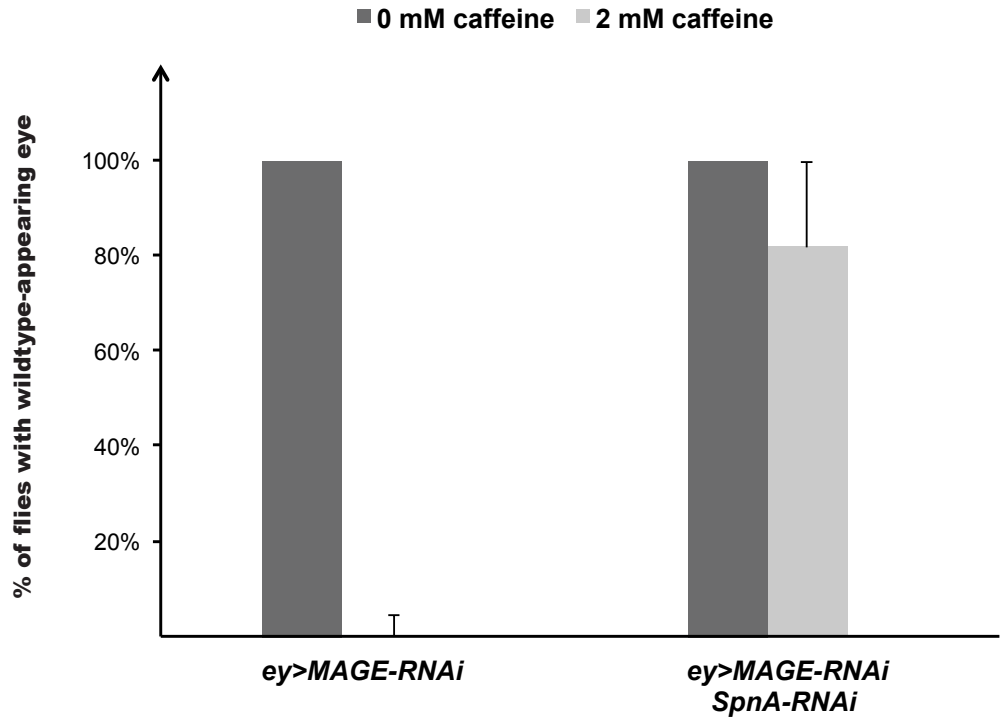

Figure 2.15. Rad51 (*Spn*A-RNAi) depletion rescues the *MAGE*-RNAi caffeine-sensitive eye phenotype.

Bars represent the percentage of flies with wildtype eye phenotypes among MAGE knockdown (UAS-Drc2/+; UAS-MAGE-RNAi/+) and MAGE Rad51 double knockdown (Drc2/+; UAS-MAGE-RNAi/UAS-SpnA-RNAi) flies that were reared on either standard media or media containing 2 mM caffeine. Data were collected from 4 replicates of each cross. Absence of error bar indicates flies of this genotype had consistent phenotypes. Done by RZ.

2.4 Discussion

In a genetic screen for mutations conferring caffeine sensitivity in flies, we identified viable alleles of *Drosophila Smc6* (*jnj*; *CG5524*) and *MAGE* (*sst; CG10059*) as well as an unknown gene (*ddt*). Additional loss-of-function alleles created by imprecise P-element excision of *Smc6* (jnj^{XL}) or targeted knockout of *MAGE* (sst^{XL}) were also viable under normal conditions, but exhibited caffeine-sensitive lethality. Although no molecular lesions were identified for most *jnj* (*Smc6)* alleles, transcript levels were dramatically reduced in all these mutants when hemizygous, implying that either mutations in regulatory regions affected expression, or that transcripts have mutations in a yet unidentified exon of the *Smc6* gene. There was no detectable *MAGE* expression in homozygous, transheterozygous, or hemizygous *sst* mutants. Furthermore, a genomic *MAGE* transgene restored expression and rescued the caffeine-dependent lethality of *sst* mutants. Loss of *Smc5* by P-element insertion also resulted in caffeine sensitivity. These genetic results as well as biochemical data showing physical interactions among SMC6, MAGE, Nse1 and Nse4 indicate that the *Drosophila* Smc5/6 complex is structurally and functionally conserved between yeast and flies. Our screen only covered one chromosome arm (3R) to obtain seven alleles of *Smc6* and two alleles of *MAGE*, representing ~20% of the genome. Homologs of the remaining SMC5/6 components reside on chromosome arms 2L and 3L (Table 2.4) and were thus not discovered in our screen. As there are no known *Smc5/6* homologs mapping to the *ddt* locus located in the 98E region of chromosome 3, identifying this gene and screening remaining chromosome arms for mutations

conferring caffeine sensitivity may lead to novel Smc5/6 components or other pathways in which *Smc5/6* is involved.

The SMC5/6 complex has been intensively studied in yeasts and human cells for its roles in chromosome replication, segregation and repair of DNA double strand breaks by homologous recombination (*81*). Depletion of Smc5 or Smc6 in *Drosophila* tissue culture cells resulted in heterochromatin bridges in 50% of mitotic cells (*47*), suggesting that the *Smc5 or Smc6* genes would be essential for viability. On the contrary, we found that the loss of *Smc5, Smc6*, or *MAGE* did not result in lethality *in vivo*, and indeed homozygous mutant flies have been maintained for generations (data not shown). There was a slight reduction in hatching rates among null eggs from null mothers in some of the mutant lines, so we cannot rule out a contribution of the maternal RNA to viability in early development. We also did not observe DNA links between sister chromatids, excess aneuploidy, or translocations in mitotic chromosomes of neuroblast squashes from Smc5/6 mutant flies (data not shown). Homologs of *Smc5* and *Smc6* in *Caenorhabditis elegans* are also dispensable for viability, however the homozygous mutant strains were prone to sterility and germ cell defects because of compromised inter-sister chromatid recombinational repair and excessive germ cell apoptosis (*22*).

In both *S. cerevisiae* and *S. pombe*, genes encoding SMC5/6 and Nse1-4 are essential and hypomorphic mutants are sensitive to genotoxic agents (*16*). In *C. elegans*, *smc-5* and *smc-6* mutant germ cells are also hypersensitive to IR and exhibit increased germ cell apoptosis even without IR exposure (*22*). In

vertebrates, *Smc5*-deficient chicken DT40 cells are sensitive to MMS and IR (*23*). Interfering with the function of human *NSE2* by RNAi sensitizes HeLa cells to MMS-induced DNA damage (*42*). The *Smc5, Smc6* and *MAGE* mutants described here are also sensitive to IR (40 Gy), HU (4 mM to 8 mM), camptothecin (0.025 mM) and MMS (0.05-0.015%), consistent with an evolutionarily conserved role in resistance to genotoxic agents. Components of the Smc5/6 complex may be responsible for existing *Drosophila* mutagen sensitive (*mus*) mutants (e.g. (*82*)) or may not yet be represented among these collections so represent novel genes important for mutagen resistance.

Our experiments suggested that cells located just before the morphogenetic furrow in the imaginal eye discs of larvae lacking Smc5/6 components were most sensitive to caffeine (Figure 2.8). Many of these cells normally become synchronized in G1 phase by being forced through mitosis by induction of the $Cdc25^{stg}$ gene suggesting that the *Smc5/6* and *MAGE* mutants described here are particularly sensitive to mitotic kinase Cdk1 activity when treated with caffeine (*69*). G2/M checkpoint responses to DNA damage and the S-phase checkpoint induced by stalled replication forks were both intact in *Drosophila Smc6* or *MAGE* mutants, however. These results may be explained by accumulating evidence that yeast *Smc5/6* mutants undergo normal initiation of the checkpoint response but then fail to repair DNA damage and recover after genotoxic stress, leading to formation of DNA bridges and aberrant mitosis (*18, 26, 79, 83*). Consistent with this explanation, *Drosophila MAGE* and *Smc6* mutants genetically interact with *ATM* and *ATR* to increase the severity of the

caffeine-induced rough eye phenotypes (Figure 2.12). Similar dependencies were also recently reported for *S. cerevisiae,* where *Nse2* mutants deficient in SUMO ligase activity were viable but needed Mec1 kinase (ATR) to survive, even in the absence of genotoxic stress (*31*).

We propose a two-hit mechanism explaining why caffeine and loss of *Smc5/6* synergistically decrease cell survival (Figure 2.16). Endogenous DNA damage can give rise to single or double stranded breaks. ATM/ATR kinases respond to these DNA breaks, coordinating cell cycle checkpoint and Rad51-mediated homologous recombination repair. Rad51 recruitment is tightly regulated to prevent homologous recombination from causing chromosome damage. Downstream effectors of ATM and ATR, such as the RecQ helicases, Smc1/3 complexes, and the Fanconi anemia complex proteins including the breast cancer susceptibility protein Brca2, all regulate Rad51 by controlling its recruitment to chromatin (Figure 2.15) (*19, 51, 84-86*). In *Drosophila,* Smc5/6 complexes regulate Rad51 activity by inhibiting precocious Rad51 recruitment during double strand break repair of heterochromatin (*47*). Our results show that loss of *Rad51* relieved caffeine-dependent defects in *MAGE* and *Smc6* mutants (Figure 2.12). This suggests that mutants lacking *Smc5/6* use other *Rad51* regulatory pathways to prevent aberrant homologous recombination, for example involving the Blm helicase (*87*). These alternate pathways are compromised by caffeine, rendering cells sensitive to endogenous genotoxic stress. Our model proposes that caffeine treatment or loss of Smc5/6 alone may affect homologous recombination repair,

but do not disrupt control of Rad51 because of the redundant regulatory mechanisms shown in Figure 2.16. However, when ATM/ATR kinases are partially inhibited by caffeine in *Smc5/6* mutants, functionally redundant Rad51 regulatory mechanisms are impaired sufficiently to allow illegitimate homologous recombination that can trigger apoptosis. As well, ATM/ATR kinase pathways could directly regulate Smc5/6 complex. This possibility is supported by evidence that Smc5/6 is loaded onto chromatin by the Mre11 component of the MRN complex that interacts with ATM (*77*), and data indicating that *Drosophila* ATR regulates the heterochromatin repair pathway upstream of Smc5/6 (*47*). In human cells in which hMMS21 (Nse2) is depleted by RNAi, ATM/ATR kinases are hyperactivated, indicating a potential feedback mechanism between ATM/ATR and Smc5/6 (*42*). Further experimental data are required to elucidate the functional and physical interactions among ATM/ATR, Smc5/6 and associated recombination repair proteins.

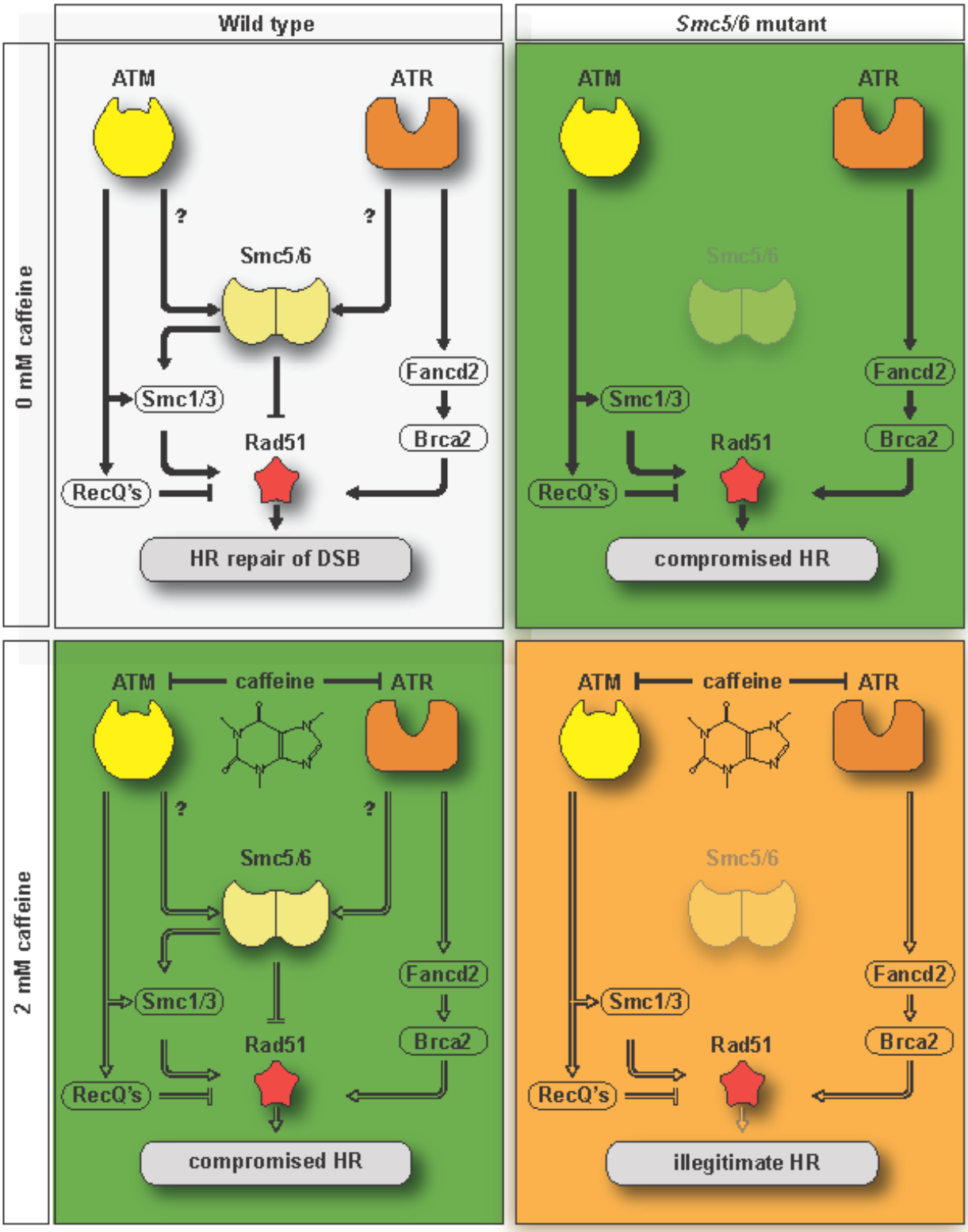
Wild type
Smc5/6 mutant
0 mM caffeine
2 mM caffeine
ATM
ATR
caffeine
?
Smc5/6
Smc1/3
Fancd2
Brca2
Rad51
RecQ's
HR repair of DSB
compromised HR
illegitimate HR

Figure 2.16. Model accounting for how caffeine and loss of *Smc5/6* synergistically mis-regulate Rad51 to cause apoptosis in *Drosophila*.
DNA double-stranded breaks are dealt with by the ATM/ATR signaling pathways and can be repaired by Rad51-mediated homologous recombination repair. Rad51 recruitment is tightly regulated to prevent homologous recombination from causing chromosome damage, as illustrated in the first panel and described in the text. Since the possibility that ATM or ATR could also regulate Smc5/6 has not yet been determined, this potential interaction is denoted by a question mark. We propose that caffeine treatment (by partial inhibition of *ATM* and *ATR*) or loss of *Smc5/6* each influence Rad51 recruitment but that these regulatory mechanisms are functionally redundant, allowing cells to survive intrinsic DNA damage (one-hit scenarios, green background). However, when the loss of *Smc5/6* and caffeine-treatment are combined, mis- regulation of Rad51 causes illegitimate homologous recombination and apoptosis, causing lethality (two hit scenario, orange background). Done by RZ.

Studies of protein complexes that are critical for cellular responses to genotoxic stress are also highly relevant to cancer therapy in humans. It is increasingly apparent that the gene expression signature of each tumor dictates in part the success or failure of chemotherapeutic treatment or radiotherapy (*88*). The expression of human Type I MAGE genes is commonly dysregulated in cancer cells. Moreover, many studies have correlated the levels of expression of particular MAGE genes with therapeutic response, prognosis and probability of metastasis (*89*). The unexpected synergy between caffeine and loss of SMC5/6 activity could potentially be exploited for new therapeutic strategies where one could preferentially sensitize checkpoint-compromised cancer cells to apoptosis. Although the therapeutic potential of caffeine for causing premature chromosome condensation in G1 checkpoint-compromised cancer cells has long been recognized, the concentrations needed to fully inhibit ATR kinases are toxic (*90*). In cells exposed to UV-light, caffeine inhibits rescue of stalled replication forks by translesion DNA synthesis, causing a switch to homologous recombination that can result in chromosomal aberrations (*91, 92*). Further studies are needed to elucidate the relationships among MAGE proteins, Smc5/6 components, and

proteins such as ATM and ATR that are also important for resistance to genotoxic agents in normal and cancer cells. In turn, mechanistic understanding of how cells respond to genotoxic stress will aid in the selection and dose of chemotherapeutic agents that target specific disruptions to DNA damage response pathways, in order to improve cancer prognosis and survival.

Table 2.1. *sst* caffeine sensitivity can be rescued by a *MAGE* transgene

Genotype	0 mM caffeine	2 mM caffeine
3Kb+MAGE/+;sst^{XL}/sst^{XL}	64	118
3Kb+MAGE/+; sst^{XL} /TM3, Ser, ActGFP	59	77
CyO/+; sst^{XL} /sst^{XL}	52	0
CyO/+; sst^{XL} /TM3, Ser, ActGFP	77	30

All genotypes were produced from the cross *sst^{XL} / sst^{XL}* X *3Kb+MAGE/CyO; sst^{XL} /TM3, Ser, ActGFP* Done by FDC, RZ, XL.

Table 2.2 P-element excision of *P{GSV1}GS3245* and *P{GSV6}GS14577* produce both caffeine-sensitive and -insensitive lines

Table 2.2 A									
Excision alleles	Caffeine sensitivity	*Smc5*^{P14577}/Smc5^{P14577} X Smc5^{exP14577}/TM3,Sb				*Smc5*^{exP14577}/Smc5^{exP14577} X Smc5$^{Df(3L)BSC418}$/TM6,Sb			
		Standard media		Caffeine 1mM		Standard media		Caffeine 1mM	
		Smc5exP14577/Smc5P14577	*Smc5P14577/TM3,Sb*	*Smc5exP14577/Smc5P14577*	*Smc5P14577/TM3,Sb*	*Smc5exP14577/Smc5Df(3L)BSC418*	*Smc5exP14577/TM6,Sb*	*Smc5exP14577/Smc5Df(3L)BSC418*	*Smc5exP14577/TM6,Sb*
1	No	50	56	18	27	61	97	51	57
2	No	28	33	14	18	60	81	30	47
3	Yes	58	79	0	16	51	30	0	11
4	No	62	101	19	35	30	33	29	36
5	Yes	N/A	N/A	N/A	N/A	42	56	0	12
7	Yes	52	77	0	22	39	40	0	8
9	Yes	24	19	N/A	N/A	41	56	1	24
10	Yes	29	40	0	2	45	47	0	16
11	Yes	57	53	0	43	38	47	1	24
12	No	17	30	15	13	63	77	26	39
13	No	54	90	28	35	33	43	14	35
14	Yes	54	61	0	21	29	32	0	33
15	Yes	52	74	0	20	82	84	11	27
Original Allele		*Smc5*^{P14577}/Smc5^{P14577} X Smc5^{P14577}/TM3,TM3,Ser,ActGFP				*Smc5*^{P14577}/Smc5^{P14577} X Smc5$^{Df(3L)BSC418}$/TM6,Sb			
		Smc5^{P14577}/Smc5^{P14577}	*Smc5^{P14577}/TM3,Ser,ActGFP*	*Smc5^{P14577}/Smc5^{P14577}*	*Smc5^{P14577}/TM3,Ser,ActGFP*	*Smc5^{P14577}/Smc5$^{Df(3L)BSC418}$*	*Smc5^{P14577}/TM6,Sb*	*Smc5^{P14577}/Smc5$^{Df(3L)BSC418}$*	*Smc5^{P14577}/TM6,Sb*
	Yes	58	77	0	33	44	56	0	7

Table 2.2 B

Excision alleles	Caffeine sensitivity	*Smc5*^{P3245}/Smc5^{P3245} X Smc5^{exP3245}/TM3,Sb				*Smc5*^{exP3245}/TM3,Sb X Smc5$^{Df(3L)BSC418}$/TM6,Sb			
		Standard media		Caffeine 1mM		Standard media		Caffeine 1mM	
		Smc5exP3245/Smc5P3245	*Smc5P3245/TM3,Sb*	*Smc5exP3245/Smc5P3245*	*Smc5P3245/TM3,Sb*	*Smc5exP3245/Smc5Df(3L)BSC418*	*Smc5exP3245 or Df(3L)BSC418/TM6 or TM3,Sb*	*Smc5exP3245/Smc5Df(3L)BSC418*	*Smc5exP3245 or Df(3L)BSC418/TM6 or TM3,Sb*
1	No	10	32	13	34	46	82	27	45
2	No	15	72	17	41	57	119	22	45
3	No	19	53	6	46	N/A	N/A	N/A	N/A
4	No	39	115	6	39	29	64	12	44
5	No	37	128	17	49	30	117	15	48
6	No	50	108	25	40	56	109	18	28
7	No	20	72	31	52	43	64	24	28
8	No	34	106	0	69	37	95	0	34
9	Yes	27	155	0	73	55	116	0	36
10	Yes	44	105	26	30	57	87	16	50
11	No	42	127	22	56	35	147	10	39
12	Yes	20	77	0	51	30	110	0	30
13	Yes	34	84	0	48	11	33	0	33
Original Alleles		*Smc5*^{P3245}/Smc5^{P3245} X Smc5^{P3245}/TM3,TM3,Ser,ActGFP				*Smc5*^{P3245}/TM3,Sb X Smc5$^{Df(3L)BSC418}$/TM6,Sb			
		Smc5P3245/Smc5P3245	*Smc5P3245/TM3,Ser,ActGFP*	*Smc5P3245/Smc5P3245*	*Smc5P3245/TM3,Ser,ActGFP*	*Smc5P3245/Smc5Df(3L)BSC418*	*Smc5P3245 or Df(3L)BSC418/TM6 or TM3,Sb*	*Smc5P3245/Smc5Df(3L)BSC418*	*Smc5P3245 or Df(3L)BSC418/TM6 or TM3,Sb*
	Yes	30	71	0	52	39	96	0	46

Done by XL

Table 2.3. Caffeine sensitivity of *MAGE* and *Smc6* double mutants is similar to sensitivity of fly mutant for *Smc6* alone

Cross	Genotype	Mutant status	No caffeine	Caffeine	Sensitivity ratio
1	sst^{XL}, jnj^{X1}/sst^{XL}, jnj^{R1}	Double Mutant	104	9	0.07
	sst^{XL}, $jnj^{R1\ or\ X1}$/*TM3,Ser,ActGFP*	Double Het	226	267	
2	+, jnj^{X1}/sst^{XL}, jnj^{R1}	*Smc6* Mutant	224	8	0.05
	+, jnj^{X1}/*TM3,Ser,ActGFP*	*Smc6* Het	238	189	
3	sst^{XL}, +/sst^{XL}, jnj^{R1}	*MAGE* Mutant	279	83	0.34
	sst^{XL}, +/*TM3,Ser,ActGFP*	*MAGE* Het	310	274	

Caffeine sensitivity of *MAGE* and *Smc6* double mutants or single mutants was tested using media with 0.25 mM caffeine. A Sensitivity ratio was calculated by dividing the ratio of the homozygous versus the heterozygous flies surviving on media containing caffeine by the ratio of homozygous to heterozygous flies surviving on standard media. A Sensitivity ratio of 1 indicates that caffeine has no effect. Double Mutant: both *sst*/*MAGE* and *jnj/Smc6* inactivated; Double Het: *MAGE* and *Smc6* heterozygous mutant; Smc6 Mutant: *Smc6* inactivated; MAGE Mutant: *MAGE* inactivated; Het: heterozygous for either *MAGE* or *Smc6*. Done by XL.

Table 2.4 Genes encoding Smc5/6 complexes in different model organisms

component	*Drosophila* CG	*Drosophila* symbol in FlyBase	*Schizosacchro myces pombe* gene	*Saccharomyces cerevisiae* gene	Human gene
Smc5	CG32438	Dmel*Smc5*	Spr18	YOL034W/SMC5	SMC5L1 (alias KIAA0594, SMC5L1)
Smc6	CG5524	Dmel\CG5524	Rad18	YLR383W/SMC6 Rhc18	SMC6L1 (alias FLJ22116, FLJ35534, SMC-6, SMC6L1, hSMC6)
Nse1	CG11329	Dmel\CG11329	NSE1	YLR007W/NSE1	NSMCE1 (alias HSPC333, NSE1)
Nse2	CG13732 CG15645	*Dmel\qjt Dmel\cerv	NSE2	YEL019C/MMS21	NSMCE2 (alias C8orf36, FLJ32440, MMS21, NSE2)
Nse3	CG10059	Dmel*MAGE* (alias dMAGE)		YDR288W/NSE3	All MAGE genes NDNL2 (alias HCA4, MAGEG1, MAGEL3, NSE3, NSMCE3)
Nse4	CG13142	Dmel\CG13142	Rad 62	YDL105W/NSE4 (alias Qri2)	NSMCE4A (alias NSE4APP4762, C10orf86, FLJ20003, NSE4A) NSMCE4B (alias SE4B,EID3)/EID1

* used by Chiolo I, Minoda A, Colmenares SU, Polyzos A, Costes SV, Karpen GH. 2011. Double-strand breaks in heterochromatin move outside of a dynamic HP1a domain to complete recombinational repair. Cell 144:732-744. SMC= structural maintenance of chromosomes; Nse= Non-SMC element protein. Compile by XL and RZ

Table 2.5 *mei-41/ATM* and *jnj/Smc6* double mutants have normal viability

	$jnj^{X1}/jnj^{Df(3R)Exel6198}$	$jnj^{X1}/TM3,Ser,ActGFP$
$mei\text{-}41^{D3}/Y$	99^{ns}	79^{ns}
FM7/Y	22^{***}	35^{***}
$mei\text{-}41^{D3}/+$	118^{ns}	110 (double heterozygotes)
FM7/+	118^{ns}	105^{ns}

jnj^{X1} homozygous males were crossed to $mei\text{-}41^{D3}/FM7;\ jnj^{Df(3R)Exel6198}/TM3,Ser,ActGFP$. The progeny representing the eight possible genotypes were counted. The number of progeny for each genotype was compared with the number of progeny heterozygotes for *mei41* and *Smc6* ($mei41^{D3}/+$; $jnj^{X1}/TM3,Ser,ActGFP$) using a chi-square test, with equal numbers expected in each category. "ns" indicates the number of progeny was not significantly different from the number of double heterozygotes ($P > 0.05$) while "***" indicates the number of progeny was significantly different from the number of double heterozygotes ($P < 0.001$). Fewer *FM7/Y* progeny survived, independent of *jnj* genotype, presumably because of non-balanced mutations on the *FM7* chromosome that reduce viability. Done by XL.

Appendix (All experiments described below are done soly by RZ):

Table 2.6 List of Long range PCR primers for *jnj*, *sst* and *ddt* regions

***jnj* regions**	**Primers**	**Sequence**	**estimated fragment size(bp)**	**0.0175pmol to ng for NEXTGen Seq**
1	jnj-F-58	CGAACAACAATAGGAGCTATCTGTGGGC	9026	104.25
	jnj-R-9084	GACACCGGGTTATTGGCACTTTGA		
2	jnj-F-4999	ATCCCATCTCACTGTTGGCTCTGC	10860	125.43
	jnj-R-15859	CTTGGGCTACGGAGCAGCAGAAAC		
3	jnj-F-10504	CATTAACCGATGCCAACGATTCCC	8774	101.34
	jnj-R-19278	GGGTGATAGGAAGGGAATGGAAGG		
4	jnj-F-14233	GGCAACAGCGAAACAGCGAATCAG	10688	123.45
	jnj-R-24921	GCCCGCACCTCAATTACCTCATCC		
5	jnj-F-19758	AAGACTGGAACGCAGACTCAAACG	11508	132.91
	jnj-R-31266	AAGAGGAGCAAACAGCAGTTCGTG		
***sst* region**				
1	sst-F-747	CTCCACGGTGCTAAAGTCCTGTCT	10082	116.45
	sst-R-10829	GGTGTAGCAGGCTGAACAAGTGAA		
2	sst-F-4811	CGCCATGCGAAGCTAATGTGATGT	9720	112.27
	sst-R-14531	GTTAGCCAGCACAGCCTTACACCC		
3	sst-F-10138	CGGAGTGTTTACTGCTTAGGTGATGTGC	9293	107.33
	sst-R-19431	CTCCACTCCGCTACCACCGATTTA		
gf	sst-F-19382	GCGCGCTAATTGAGGATTTCAGGTGAGC	1265	14.61
	sst-R-20647	TGAATCCGCAAAAGGAGATACCCGTGCTGG		
5b	sst-F-20640	GTCGTCGTCCAGCACGGGTATCTC	9319	107.63
	sst-R-29959	TCGGTGCTATTACAGTCACTGGTC		
6	sst-F-25815	TCTTCCTATGCGTCCTCCACAACC	8671	100.15
	sst-R-34486	CCACCTGCTGTTCGTTGGTAATGTCT		
7	sst-F-30331	CCAGCCAATAGAGTAACGACAACG	9735	112.44
	sst-R-40066	GTAATCAGGTGCGGTTTCTTCTCC		
8	sst-F-34742	CAAGGAAGTGGGTGCTTAGGTCTG	11501	132.84
	sst-R-46243	GGCATTTACCCAGTCAAGTCCTCC		
9	sst-F-40040	CTTGGAGAAGAAACCGCACCTGAT	10295	118.91
	sst-R-50355	CAGTAAGTTGTGGCAGATGGAGGC		
10	sst-F-46682	GGCAGAAGCAATCTTGAATCTCGTGG	9070	104.76
	sst-R-55752	GGAGTGATGGTCCTTTCAGGTGGC		
11	sst-F-50371	GCTGCGATGCCTGAATCTTGGTGT	10488	121.14
	sst-R-60859	CGAGCGTATGAGCAATGTCCTAAAGC		
12	sst-F-55459	TCCTCGTTCCATTCGCATCGTCTT	11616	134.16
	sst-R-67075	AGGAAGACAGACAAAGGGTGAGGG		

ddt region				
1	ddt-F-194	GGCATTCACTTGTCTCCTTGTCTC	10239	118.26
	ddt-R-10433	GGTAGATGGTTCAGTTCCGTTTCG		
2	ddt-F-5742	CATCTTGTTCCATTCCGAGTGCTACG	8928	103.12
	ddt-R-14670	CCCTCCATTCCCAGTCTTAGTCCC		
3	ddt-F-11034	GCAACTTTAGCCACTTCAGCCACT	8647	99.87
	ddt-R-19681	GAGCACTCAACACCCAACACTTTC		
4	ddt-F-15730	GTCTATTGACCATTGACCACCTGC	9699	112.02
	ddt-R-25429	AGACGAACTTTCCTAACGACCCTC		
5	ddt-F-19381	TTAGTCAGTGGGTGAGTGAGGGTG	10202	117.83
	ddt-R-29583	CACAAGAACTCACCTCAGCACCAC		
6	ddt-F-25407	AGGGTCGTTAGGAAAGTTCGTCTG	9852	113.79
	ddt-R-35259	CCTGGAGTATGCCGTTGATGACCT		
7	ddt-F-29434	ATGTGCTTTGGCTTCCTCATCTCC	13194	152.39
	ddt-R-42628	CCATTCCACTTCCTCCTCATCTGC		
8	ddt-F-34924	CAACAGCAGCCGTAAGTTTCCCTC	11883	137.25
	ddt-R-46807	GGTTGGTGGTTGTGAAATGCCCTA		
9	ddt-F-40295	CGCTTACGCTTTCGCTTACCTCCT	11417	131.87
	ddt-R-51712	CGATGGATACCCGAGAAGTGAATGGA		
10	ddt-F-45557	AATCTGCCCACCCATTTCCCTTCA	10479	121.03
	ddt-R-56036	AGAGCGTCTTGTTCCGACTCCTGT		
11	ddt-F-50071	ACAGACACGAATACCGACCATCCT	12308	142.16
	ddt-R-62379	CCATCCTCCCATCTCCATTCCAGTTT		
12	ddt-F-56388	AGTCATTTGTCAGTACGACCCAGGAG	10478	121.02
	ddt-R-66866	GCCGAGCCTGAGAATGGAATGAAA		
13	ddt-F-61150	GGGTCTCAAACTCCTGCGACTACAT	9113	105.26
	ddt-R-70263	CCACCCACGCAGTCTACTTTCATTCA		
14	ddt-F-67513	CATTAGGTTCAAGGTTAGCCAGTGCG	10155	117.29
	ddt-R-77668	TGGGCAGTCGTAAGTCGTAACAAGC		
15	ddt-F-70268	CCACTTTCATTCGTTCCGCTTGCT	11666	134.74
	ddt-R-81934	GCATTTCCAATCCAAGTCCGTAGAGG		
16	ddt-F-77552	GCCGCTTGTTACGACTTACGACTG	9310	107.53
	ddt-R-86862	ATTCCATTACCCGTGGCGAAACTC		
17	ddt-F-81135	TCACATCCATCCACATCCGCTTTC	10199	117.8
	ddt-R-91334	GGACATTCCAAGGAGCCTGTAGTT		
gf	ddt-F-91045	GGGGACTTGGTTAGTTGAATAGCA	911	10.52
	ddt-R-91956	TCGTATCCAACTACATTGTCACCC		
18a	ddt-F-87364	TTGGTTAGTGTCCGAGTTTGCTGC	10201	117.82
	ddt-R-97565	CCCAAATAATCTTCGGCACTCCAC		

19	ddt-F-91474	CGGCGTCGTTTGTAGTTCATTTGG	10534	121.67
	ddt-R-102008	CGTCGAACCGTCTGTATCTGAGTA		

Each mutant region (which is broken down into overlapping small regions about 10 kb in size) includes the deficiency-mapped genomic interval and 10 kb to each side. The numbers in the primer names correspond to the genomic coordinates. note: gf-2, gf= gap filling. The generated long range PCR products were then used as templates for Illumina NextGen re-targeted sequencing.

Table 2.7 Smc5/6 qPCR primer list

The first 5 pair of primers are for canidate genes in the deficiency mapped *jnj* region (Fig 2.5). *P38c* and *Mpk2* are kinase candidates near the predicited *jnj* region, which is also where *CG5524* is (Append Fig 2.17). MAGE primers were used to measure *MAGE* expression levels in *sst* mutants (Append Fig 2.21). CG33346 to CG10000 are candidate genes in the *ddt* region (Append Fig 2.23)

Genes	Primer names	Sequence
CHORD	qCHORD-110F	ccgaaacttaataatcatggaaca
	qCHORD-110R	tggtgccgacaagattcat
CG5515	qCG5515-RA#149F	cgcctctcagctgtaagtaacc
	qCG5515-RA#149R	tttcaacaaacaaataagacaaatcc
	qCG5515-RB#91F	cctcaaatggaagctcgaat
	qCG5515-RB#91R	cattgttcttctcccgcttg
CG6204	qCG6204-70F	gctatattgtagacggccagatg
	qCG6204-70R	ctaagcttaactcctggtttcatgt
CG5524	qCG5524-54F	aatggttcagtttcagtttgagc
	qCG5524-54R	atcttccacgtcttctccttgt
CG13606	qCG13606 (A/B) - 13F	ttaatcagccagcaaatatgaaat
	qCG13606 (A/B) - 13R	actggcccagaaagtattgc
P38c	qP38c-58F	cccgaaaaacgtatcacagc
	qP38c-58R	gctcaataagatcccgaaggt
Mpk2	qMpk2-120F	aagcatatggatcatgagaacg
	qMpk2-120R	attagcgggatgtggatgg
MAGE	qMAGE-#4F	cgcagctaggagccagaat
	qMAGE-#4R	accacatcgacgggttgt
CG33346	qCG33346F-#154	tactgcctgccccagaact
	qCG33346R-#154	ttggatcacagcagaaggtg
CG9989	qCG9989F-#138	ggcttatcgctacgtaaacacc
	qCG9989R-#138	cttccctcgtaggcattgtc
CG9990	qCG9990F-#108	gtgcccaagggaacaatcta
	qCG9990R-#108	catgtaacgacgtccaacga
htt	qhttF-#129	cgaggacattgtctgcacttat
	qhttR-#129	gtcgccgagcagatgtaaa
AR-2	qAR-2F-#68	gccttcctctccgagaattt
	qAR-2R-#68	cggcaaatcagatgtgtagttc
CG10000	qCG10000F-#42	atggcagtcagcgagatgt
	qCG10000R-#42	gatatctcgacccgaacacc
CG9997	qCG9997F-#5	gttcacgacgaacgcatct
	qCG9997R-#5	cgtgtaggcgggacagtt
CG14061	qCG14061F-#119	agtgaagaagtcgcccaatg
	qCG14061R-#119	tcatcgaatgctaattcctctg
CG34295	qCG34295F-#144	gtgcctgcaagacaacattc
	qCG34295R-#144	gcacacagcatactgggagtt
new CG10000	qCG10000-#20F	cccaggaaacctatctgcac
	qCG10000-#20R	cggtctcagtgcgtagaaca

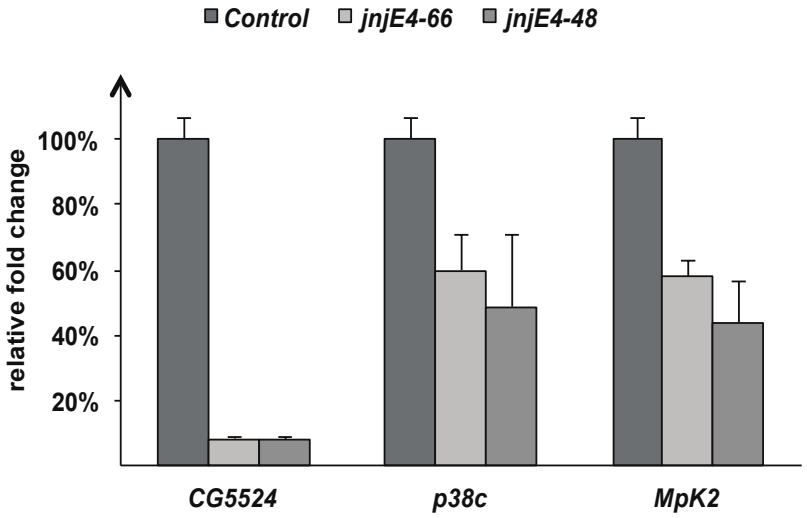

Figure 2.17. Candidate gene *CG5524*, *p38c* and *MpK2* expression levels in jnj^{E4-48} and jnj^{E4-66}
jnj^{E4-48} and jnj^{E4-66} are two of the seven *jnj* alleles chosen as examples based on their relatively stronger caffeine EGUF eye phenotypes (data not shown). jnj^{E4-48} also had occasional homozygote survivors that were sterile. *CG5524* encodes the *Drosophila* homolog of *Smc6*. P38 MAPKs are stress-induced mitogen-activated protein kinases. *Mpk2* is also called *p38a*; it is found next to *p38c* in the predicted *jnj* genomic region. Testing of *p38a*, *p 38c* and *Smc6* confirmed that *Smc6* is the gene affected in *jnj* mutants.

Protocol for *CG5524*, *p38c*, *MpK2*, qPCR experiment:

Virgin flies from the strain $w^{[1118]}$; *Df(3R)Exel6198,* $P\{w^{[+mC]}$*=XP-U}Exel6198/TM6, Tb, Sb* were cross with male flies of the strain *FRT 82B,* jnj^{E4-48} *or* jnj^{E4-66}*/ TM3, Sb* (*EGUF* floating). Non -*Sb* flies were collected ($w^{[1118]}$*; FRT 82B, ,* jnj^{E4-48} *or* jnj^{E4-66}*/Df(3R)Exel6198,* $P\{w^{[+mC]}$*=XP-U}Exel6198*), flash-frozen, and stored at -80° C. mRNA was extracted using the Trizol protocol from *jnj/def* flies, as well as *Iso* flies. Three biological replicates were collected. RNA was aliquoted and stored at -80°C at a concentration of 1 µg/µl. cDNA was synthesized using the High Capacity cDNA reverse transcription kit, using 1 µl of RNA diluted 1 in 10. 1 µl of cDNA in 1 in 20 dilution was used in the qPCR experiment.

Expressions of CG5524, P38c and MpK2, were quantified with SYBR chemistry and using Rp49 as endogenous control gene.

Additional Experiments regarding *jnj:*

HNF4 RNAi Hepatocyte nuclear factor 4 (HNF4) is a member of the steroid hormone nuclear receptor family that is mostly expressed in liver, intestine and kidney and is very important for liver function and development in mice. In *Drosophila*, the homolog of HNF4 is expressed in mid-gut, fat body and Malpighian tubules and is important for gut formation (*93*). These are key detoxification tissues for xenobiotics such as caffeine. HNF4 functions as a key regulator of many metabolic pathways. HNF4 is located in the 29D region where the *jnj* mutation was discovered, thus HNF4 was tested as a candidate gene for *jnj*. *ey-Gal4, act-Gal4 and hs-Gal4* drivers were crossed to *UAS-Hnf4-RNAi* (VDRC v12692 w^{1118}*; P{GD4362}v12692*). No caffeine dependent phenotype was observed.

Dominant-interaction tests $jnj^{E4\text{-}66}$*/TM3, Sb* was crossed to sst^{RZ}*/TM3, Sb*, looking for caffeine specific lethality of the non-Sb class on 0.15 mM, 2 mM and 5 mM caffeine media in triplicates. No caffeine specific interaction between *jnj* and *sst* could be discerned. A similar test of *atm6/TM6B,Tb* X sst^{RZ}*/TM6, Tb Sb* was carried out on media plus/minus 2 mM caffeine . No dominant interaction was recognized.

***Smc6-RNAi* phenotype** As a useful tool to study tissue specific smc6 depletion, an RNAi line was obtained from VDRC (VDRC v107055 *P{KK102274}VIE-260B*). A ubiquitous knockdown of *Smc6* with a strong *Actin 5C* driver results in

complete lethality in the pupal stage on standard media, which is quite different from the mutant phenotypes where the homozygotes die specifically on caffeine media with no adult structure developed. The RNAi animal appeared to lack a proper head structure (Appendix Figure 2.18). However this defect was not observed in the jnj^{R1} homozygotes or jnj^{R1}/jnj^{X1} transheterozygotes. This begs the question whether the *jnj* alleles available are not true nulls or the RNAi phenotype was due to a non-specific secondary target effect. In order to answer this question, one could do a western blot with *Smc6* antibody (received from Dr. Karpen (*47*)) to look at Smc6 protein level in *jnj* mutant vs *Smc6*-RNAi larvae; or cross the smc6-RNAi into the *jnj* mutant background (already made by me) and look at the severity of the phenotype in the doubly affected flies.

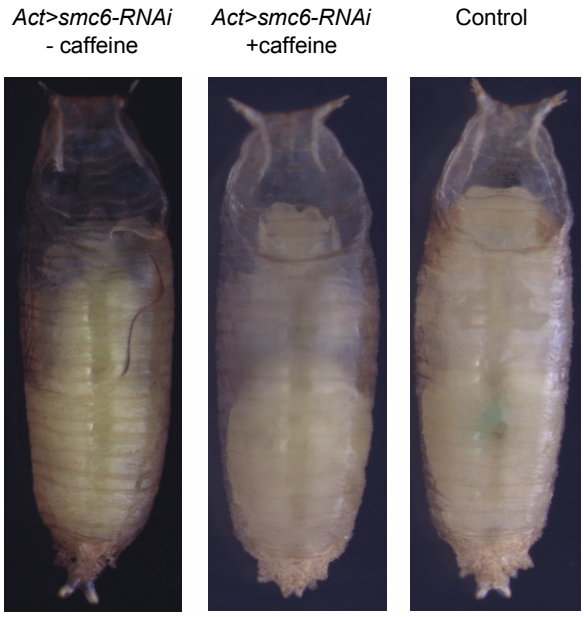

Figure 2.18. Loss of *Smc6* by RNAi is more severe in phenotype than *jnj* mutants. Ubiquitous knockdown of *smc6* with Actin5C driver resulted in caffeine independent late pupal lethality. The head structure of the RNAi fly seemed missing a large portion.

FLP-induced DSBs Affect *jnj* Mutant Viability Smc5/6 mediates HR repair of DSB in damaged DNA. To test the sensitivity of *smc6* mutants to persistent DSBs

that are unlike irradiation induced DSBs, I crossed a heat shock inducible flippase into a *jnj* transheterozygous background with the presence of FRT sites near the *jnj* alleles. Flippase binds to FRT sites and induce site specific DSBs. The *hs>flp; jnj* transheterozygotes were heat shocked repeatedly since the mid point of embryogenesis through out development till eclosion. If the DSBs were not repaired properly due to a lack of a functional *Smc5/6* complex, the persistent damage could trigger excessive apoptosis, which is detrimental to the viability of the whole organism. So I expected that the *hs>flp; jnj* mutants are fewer in numbers than the control classes. I scored for eclosion rate of two different classes of *jnj* transheterozygotes (with or without *hs>flp* present). Compared to no heat shock controls, the *hs>flp* expressing class showed a trend of decreasing viability (Append Figure 2.19).

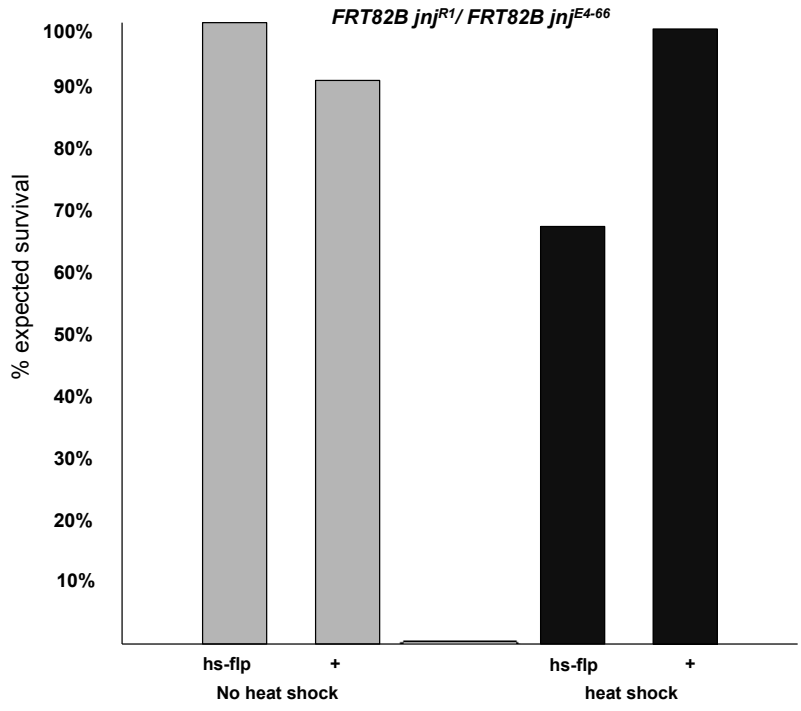

Figure 2.19. Survival of *hs>flp; FRT82B jnj^{R1}/ FRT82B jnj^{E4-66}* is compromised under heatshock condition. Number of expected adults of *jnj* transheterozygotes carrying *hs>flp* was normal without heatshock but was reduced compared with the no *hs>flp* transheterozygotes when DSB inducing flippase was turned on by heatshock.

Caffeine causes apoptosis of synchronized cells in front of MF in wild type as well as in *smc6* and *MAGE* mutants. Caffeine induced massive apoptosis in the imaginal discs of the *smc6* and *MAGE* mutants (Figure 2.8). Intriguingly, there was a dorsal-ventral stripe of apoptotic cells located immediately anterior of the morphogenetic furrow (MF) in caffeine-treated wild type discs (Append Figure 2.20), shown here the anti-activated caspase 3 is in green, DNA is visualized by hoechst staining in blue. The same stripe pattern is seen in caffeine treated *smc6* and *MAGE* mutant eye discs (Figure 2.8), only it is widened, in addition to a random pattern of apoptosis in the anterior part of eye-antennal discs. This indicates rapid cycling proliferative cells are sensitive to caffeine in a certain stage of their cell cycle. We have not fully understood this phenomenon, but it is certainly very interesting.

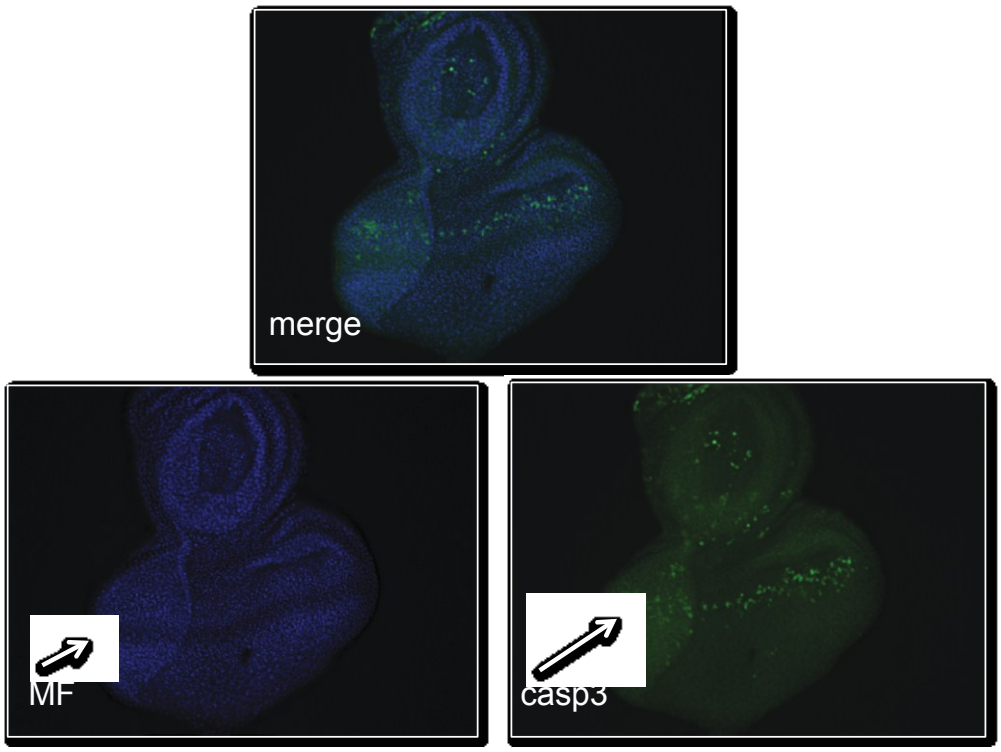

Figure 2.20. Caffeine-induced apoptosis of synchronized cells in front of the MF. Activated caspase 3 staining of caffeine treated wildtype eye-antenna imaginal discs revealed a stripe of apoptotic cells located immediately anterior of the morphogenetic furrow. Green is the anti-caspase 3 staining and blue is Hoechst staining for DNA. The MF is visualized by the Hoechst staining.

Table 2.8 PCR primers for *smc6* and *MAGE* genomic region (Also used in sanger sequencing)

Primer	Sequence	Notes
pCG5524-20018437F	TGCTGGCCCTGTTGGTTGCC	
pCG5524-20019192R	CGACCTGGTCACACTGCGCC	
pCG5524-20018838F	TCTATGAGCTTTACCTGGCAAATAC	Amplified a wrong region
pCG5524-20019504R	TTCCTTCGACTGACTGTTTAAATTC	Amplified a wrong region
pCG5524-20018844F	TTCTTCGACAAAATCCGGCTCCGCCTC	
pCG5524-20019687R	TCTGCAGCTCCAGGGACTTCAAGGACT	
		Drosophila Coordinates
pMAGE-601F	CGGTGAGCTGCGAAACCCGT	2982279
pMAGE-2586R	GCGCAGATTGGGGCCGAAGT	2980332
pMAGE-2155F	CCCTCGCAGGAAGCGCAACA	2980744
pMAGE-3340R	TCGCCGCAAGGATGGCACTG	2979578
pMAGE-3070F	AACGCGGCCGTTGGAGTAGC	2979810
pMAGE-4851R	GCGCGCAAAGATCAGCGTCG	2978066

The numbers in the MAGE primers represent the physical bp in relation to the start site obtained from FlyBase FASTA downloaded sequence. The *Drosophila* genomic coordinates are given under notes.

Table 2.9. Additional MAGE Sanger sequencing primers for mutation confirmation

Primer	Sequence	Drosophila Coordinates
sMAGE-1821F	GGCAACCAAGTGAAAAAGGATGCG	2981055
sMAGE-1374R	AGCAGACACACGCGCCGAAG	2981544
sMAGE-2490F	CGGCAACCGCATCGAGGACT	2980390
sMAGE-2786R	GCCATGGAGAGGTGATGGCCG	2980133
sMAGE-4028F	TCCTTGCGCAGCAGCTCGAT	2978852
sMAGE-4089R	CCCGGCTTGCCCCCAAAGTG	2978974
sMAGE - 2240F	AGAAGATTCCCATAAAGGACAAGG	2980729
sMAGE - 1130R	AACAAAAGCAGAATATCGGAGTTC	2979619
sMAGE - 1474F	TTAGTTTTGAAAGTTGTTTGCTTCC	2979963
sMAGE - 1731R	TGTGGGTTTGTATTGATTGATTTC	2980220

Additional data regarding *MAGE*

Mapping of *sst*^{2}, the other EMS-Induced MAGE allele. *sst*^{2} is the other allele of MAGE found in the EMS caffeine screen which is caffeine sensitive and failed to complement *sst*^{RZ} . *sst*^{2} were re-mapped with new deficiency stocks that have well defined break points (Table 2.10) and its caffeine sensitivity reconfirmed. No point mutation or small deletion was uncovered in the coding region of *MAGE* in *sst*^{2} mutants.

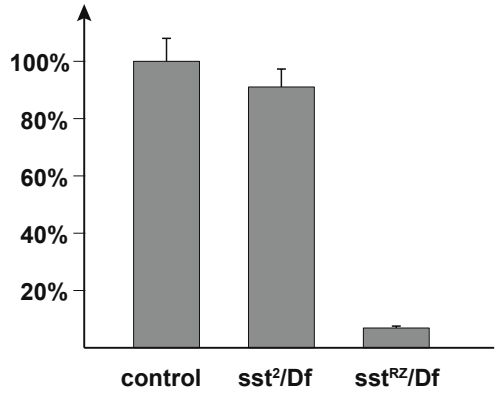

Figure 2.21. *MAGE* transcript level in *sst*^{RZ} or *sst*^{2} hemizygotes. qPCR quantification of the MAGE mRNA level in *sst*^{2} and *sst*^{RZ} hemizygotes. Control: Iso. Df: Deficiency.

Table 2.10 Deficiency stocks to map *sst*^{2}

Stock #	Symbol	Deleted Segment	Coordinates
24969	Df(3R)BSC465	84D3;84F9	3R:3132512;4127907
9076	Df(3R)ED5223	84D9;84E11	3R:3317426;3919805
26581	Df(3R)BSC729	84D14;84F5	3R:3575809;4069851
29996	Df(3R)BSC873	84E1;84F5	3R:3591219;4069851
24970	Df(3R)BSC466	84E1;85A10	3R:3657392;4573406
9622	Df(3R)BSC196	84E6;84E8	3R:3799845;3852982
9200	Df(3R)ED5220	84E6;84E11	3R:3803496;3919805
9699	Df(3R)BSC222	84E8;84F6	3R:3837757;4076143

20E rescue of caffeine specific lethality in *MAGE* mutants sst^{RZ}/Df and sst^{RZ}/sst^{XL} flies die as pre-pupae on 2 mM caffeine media with a larval-like appearance (Append Fig 2.22). One theory is that this caffeine induced developmental defect could be due to an insufficiency in ecdysone production. To test this hypothesis, I knocked down MAGE via RNAi in the key endocrine tissue of the fly, the prothroatic gland and looked for rescue of the caffeine induced lethality by supplementing the active form of ecdysone, zohydroxy-ecdysone in the fly media. To make the desired media, 200 mM caffeine stock solution and 10 mg/ml 20E solution in 95% EtOH were used. The drugs or solvents were blended into hot molten fly media and then pipetted into plates. Adobe Photoshop setting was 65.0 ms Exposure, 2.8X Gain, 0.50X Colour saturation.

1st instar larvae of the control genotype or the non-GFP *sst* hemizygotes or transheterozygotes (selected against GFP balancers) were transferred on to each of the four types of plates. Survival and phenotype of the animals were scored.

Table 2.11 Media formula used in the 20E rescue of caffeine caused motility of *MAGE* mutants

Media type	Soln. 1	Soln. 2	Total media volume
Control	2 ml 95% EtOH	0.6 ml water	60 ml
Caffeine only	2 ml 95% EtOH	0.6 ml caffeine	60 ml
20E only	2 ml 20E	0.6 ml water	60 ml
Caffeine +20E	2 ml 20E	0.6 ml caffeine	60 ml

Table 2.12 Phenotype observed in 20E rescue experiment of caffeine lethal *sst* mutants

	Iso control	***Sst***
Control	Normal adults	Normal adults
Caffeine only	Normal adults	Pre-pupa lethal
20E only	Early smaller pupa, small adults	Early smaller pupa, small adults
Caffeine +20E	Early smaller pupa, small adults	Larva-like pupa

Phm>MAGE-RNAi to knockdown *MAGE* in the prothoracic gland (PG) that secretes the hormone ecdysone gave giant pupa, which turned into giant flies (Append Fig 2.22). All of these together showed that MAGE might play a role in maintaining PG function but the caffeine lethality was not a simple developmental defect from a lack of ecdysone. MAGE protein overexpression was associated with enhanced apoptosis (*35*). Overexpressing *MAGE* ubiquitously with *Act5C-Gal4* produced complete lethality. Overexpressing *MAGE* by *ey-Gal4* gave a curious caffeine specific lethality however the viability on the standard media was reduced as well. *eyeless* is expressed in part of the brain, this could account for the observed lethality because *MAGE* is very important for neurogenesis and maintenance (*62*). *Phm>MAGE-cDNA* animals were arrested as 2nd instar on standard media and arrested as 1st instar on 2 mM caffeine. Upon dissection the

small PG tissue was hardly visible. Of course this observation needs to be carefully repeated and PG marked by a visible marker such as GFP in order to confirm. Because *phantom* has expression in the salivary gland, it was tested too. The salivary gland driver *cg* and *sgs3* crossed to *MAGE-cDNA* didn't have any visible phenotype. Taken together with the *phm>MAGE-RNAi* phenotype, MAGE seems to be required for proper PG function and the level of MAGE is critical for tissue integrity.

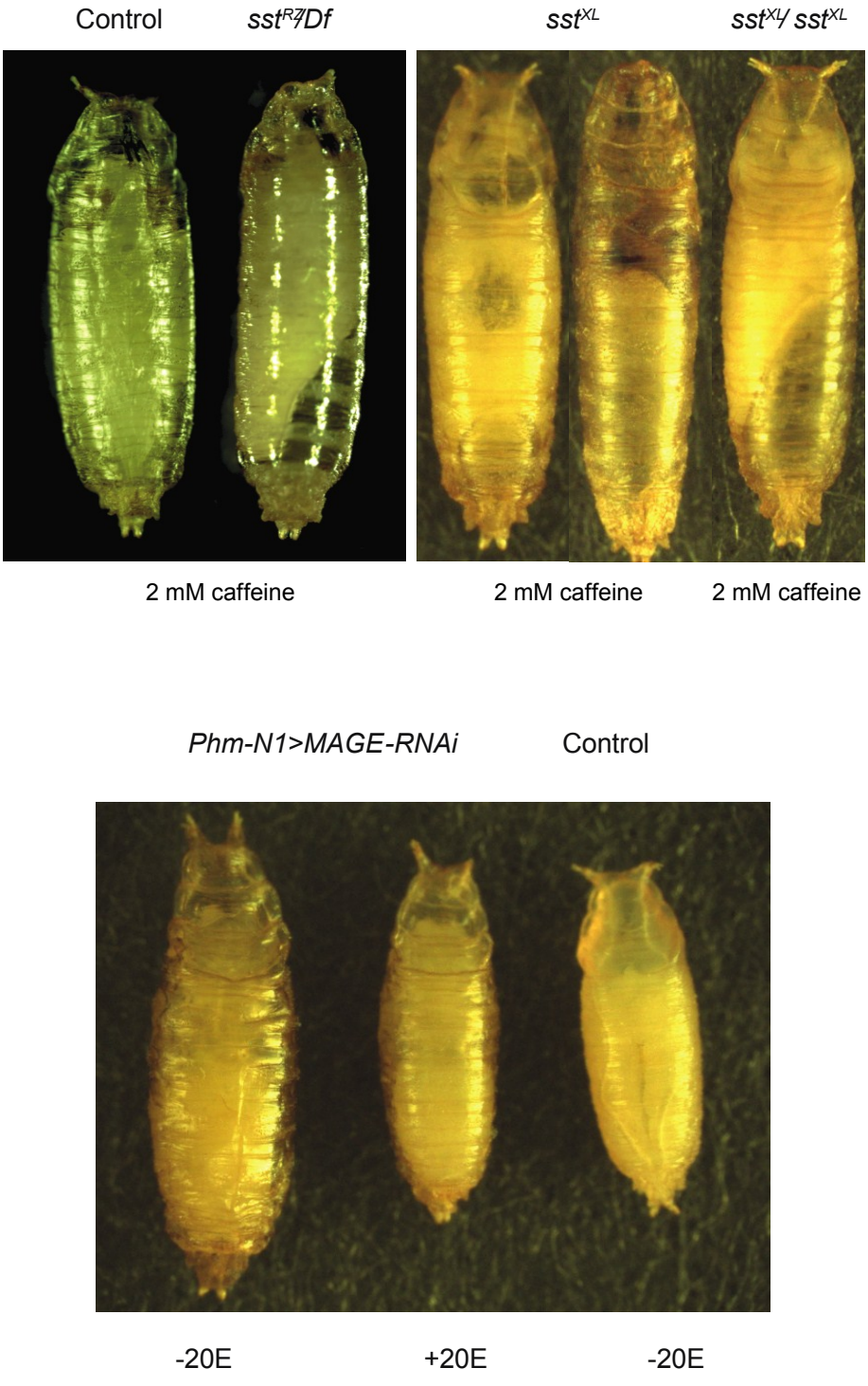

Figure 2.22. *MAGE* mutants are sensitive to 2 mM caffeine in all genetic combinations. The animals die in the pupal case without forming any adult structures. A test of *MAGE's* role in development and eclosion by knocking down *MAGE* with a prothoracic gland GAL4 driver gave giant overgrown larvae that occasionally develop into giant adults. This defect can be rescued by feeding these smc6-RNAi larvae 20 hydroxy-ecdysone (20E). However, 20E speed up the wandering and pupariation timing of caffeine-treated *MAGE* mutants but cannot rescue the lethality resulting from degeneration of the imaginal discs (data not shown).

***Smc6, MAGE* and *ddt* mutants' sensitivity to ATM inhibitor CP466722, PI3K inhibitor wortmannin and LY294002**.

The proposed molecular basis of caffeine's actions on the smc5/6 complex is through its inhibition of PI3K-like kinases ATM and ATR. I proposed that smc5/6 works downstream of ATM/ATR and caffeine specific phenotypes of the mutants are due to ATM/ATR inhibition. If that is the case, small molecule inhibitors of PIKK should recapitulate the caffeine sensitive phenotype. Now with ATM or ATR specific inhibitors, one can also try to discern which of the two kinases is largely responsible for the caffeine phenotypes, or perhaps both are equally important. CP-466722 is a potent and reversible ATM inhibitor, does not affect ATR and inhibits PI3K or PIKK family members in cells (*94*). Another related compound is KU-55933 (used by Xiao Li), which is another potent and specific ATM inhibitor that is highly selective for ATM, not ATR, DNA-PK or mTOR. Besides these highly specific inhibitors, two well-known general PI3K inhibitors wortmannin and LY294002 were used in my studies (*95, 96*). They are known to inhibit PI3K-related kinases as well. Although LY294002 is somewhat less potent than wortmannin, it is a reversible inhibitor whereas wortmannin inhibits irreversibly.

Viability of DMSO control and CP466722 treated *Iso* control or caffeine-sensitive mutants was tested and no obvious differences were found. Because eye phenotypes are more readily seen than a general viability test, I used the EGUF system to make homozygous eye mutants of *smc6*, *MAGE* or *ddt* and looked for a rough eye phenotype on drug containing media. *ddt*, jnj^{R1} and sst^{RZ} were used in

this particular experiment. CP466722 was dissolved in DMSO and test at five concentrations: 10μM, 30μM, 90μM, 300μM and 900μM. Parallel caffeine treatment was set up as positive controls. Concentrations above 30 μM CP466722 in DMSO were too high for survival of flies. It was repeated once with the lowest concentration 10 μM, the high DMSO solvent concentration in media gave a uniformed rough eye phenotype that was indistinguishable between DMSO and DMSO+CP466722. I then changed the solvent to hot ethanol to reduce the toxicity of solvent to the flies because the ethanol evaporates and is not present in the media; there again was no obvious difference at 10μM. I also reduced the DMSO concentration along with the drug (7.5μM in 0.03% DMSO) there was no rough eye phenotype any more. In the future, one could fine-tune the drug concentration between 7.5 μM and 10 μM and use a less toxic solvent than DMSO.

The same is true for wortmannin and LY294002. There was no obvious difference between eye mutants and controls. Wortmannin concentrations tested were 1.4 μM, 4.6 μM, 15 μM and 50 μM. 50μM is a lethal dose for flies. I tested lower doses: 4 nM, 13.6 nM, 45 nM, 150 nM, 500 nM and 1.5 μM again. All concentrations tested were too high for survival of adult flies. 4nM was repeated, and gave rough eye controls as well as eye mutant flies. LY294002 was tested with concentrations: 45 nM, 150nM, 500nM, 1.5μM, 45μM, 150μM, 500μM and 1.5mM. All concentrations of LY294002 contained too high levels of DMSO that resulted in either organismal death or having a non-specific rough eye phenotype.

The extremely low solubility of such kinase inhibitors poses a real challenge to study the *in vivo* effect of them in *Drosophila.*

Experiment regarding *ddt.* *ddt* was placed by deficiency mapping in 98E region of chromosome 3R. As a first easy test to gather some clues of what the *ddt* mutation might be, I tested candidate genes in the *ddt* region were assessed of their transcript level in *ddt* hemizygotes. All the data points were normalized to the highest value. The expected transcript level is 50% of that in the controls. *Htt, CG9990, CG9989, AR-2* and *CG14061* fitted the expectation. CG9997 did not show a reduction due to haploid of the locus. *CG33346, CG14061* and *CG34295* seemed to be higher in mRNA levels that the diploid control, suggesting auto-regulation events.

RNAi knock down is another approach to identify candidate genes by looking for phenocopy of mutant phenotype. Available VDRC generated RNAi lines (GD) of all ten candidate genes in the *ddt* region were tested specifically in the eye on 2 mM caffeine looking for caffeine-induced rough eye. None of the RNAi knockdowns produced a rough eye phenotype as seen in *ddt* eye mutants. Mutant alleles of htt were also tested in complementation test with *ddt*, no caffeine specific lethality was observed. *

*A version of this chapter has been published. Li X, Zhuo R, Tiong S, Di Cara F, King-Jones K, et al. (2013) ***The Smc5/Smc6/MAGE Complex Confers Resistance to Caffeine and Genotoxic Stress in Drosophila melanogaster***. *PLoS ONE 8(3): e59866. doi:10.1371/journal.pone.0059866*

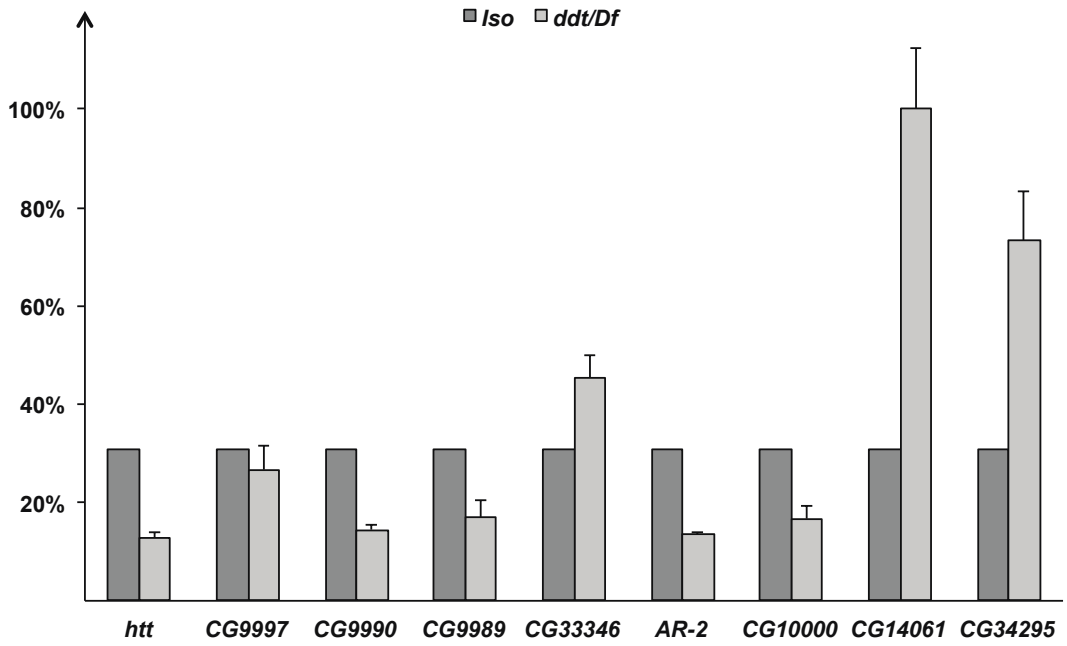

Figure 2.23. Transcript levels of *ddt* candidate genes in control and ddt/Df hemizygotes.

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Chapter 3

Analysis of metabolic insecticide resistance genes in *Drosophila melanogaster*

3.1 Introduction:

3.1.1 Insecticide modes of action and general mechanisms of insecticide resistance

Insect pests pose eminent threats to agricultural food production and human health worldwide. For instance, *Locusta migratoria*, a swarm-forming species also known as the African Migration Locust, is one of the most destructive agricultural pests worldwide. Since the dawn of agrarian civilization, locust plagues have been viewed as one of the most devastating natural disasters. Today, vector-borne diseases are a worldwide health concern. Mosquito-borne human diseases such as Dengue fever, yellow fever, HIV, malaria and West Nile viruses are a significant cause of human morbidity and mortality. Effective control of insect populations is of great economical and health importance.

Insect populations are traditionally controlled through the use of insecticides. Insecticides are often categorized by their modes of actions on arthropod physiological processes. The Insecticide Resistance Action Committee (IRAC) classifies the most common insecticides by their mode of action on various target sites in the insect (Table 3.1).

Table 3.1 MoA classification of major insecticides modified from IRAC

Main Group and Primary Site of Action	**Chemical Sub-group or exemplifying Active Ingredient**
1 Acetylcholinesterase (AChE) inhibitors Nerve action	Carbamates, Organophosphates
2 GABA-gated chloride channel antagonists Nerve action	Cyclodiene organochlorines, Phenylpyrazoles
3 Sodium channel modulators Nerve action	Pyrethroids, Pyrethrins DDT, Methoxychlor
4 Nicotinic acetylcholine receptor (nAChR) agonists Nerve action	Neonicotinoids, Nicotine, etc
5 Nicotinic acetylcholine receptor (nAChR) allosteric activators Nerve action	Spinosyns
6 Chloride channel activators Nerve and muscle action	Avermectins, Milbemycins
7 Juvenile hormone mimics Growth regulation	Juvenile hormone analogues, Fenoxycarb, Pyriproxyfen
11 Microbial disruptors of insect midgut membranes	Bacillus thuringiensis and the insecticidal proteins they produce
14 Nicotinic acetylcholine receptor (nAChR) channel blocker, Nerve action	Nereistoxin analogues
15&16 Inhibitors of chitin biosynthesis Growth regulation	Benzoylureas, Buprofezin
17 Moulting disruptor, Dipteran Growth regulation	Cyromazine
18 Ecdysone receptor agonists Growth regulation	Diacylhydrazines
*12,13,20,21,24,25 Mitochondrial function inhibitors Energy metabolism	Rotenone etc
23 Inhibitors of acetyl CoA carboxylase. Lipid synthesis, growth regulation	Tetronic and Tetramic acid derivatives
28 Ryanodine receptor modulators Nerve and muscle action	Diamides

Derived from Insecticide Resistance Action Committee (IRAC)'s Mode of Action (MoA) classification version 7.3, February 2014. To simplify, not all groups in the IRAC classifications are shown. * Groups that target different components of the mitochondria are put into one group for simplicity. For a full list, visit http://www.irac-online.org/documents/moa-classification/?ext=pdf

To summarize, current insecticides available on the market affect three major aspects of insect physiology. The earliest discovered and also biggest group is a mega-group targeting different ion channels or receptors in nerve and muscle. Famous examples include the well known DDT, pyrotheroids, pyrotherin, carbamates, organophosphates and neonicotinoids although their modes of action are on different target sites. The molecular mechanisms by which these pesticides work have been studied in great detail (*1*). Ryanodine receptor modulators appear to be a new member of this ever-expanding group. Another major group represents chemical analogues of insect hormones that disrupt insects' natural life cycle. These include juvenile hormone mimics, molting disruptors, ecdysone receptor agonists as well as inhibitors of chitin or lipid synthesis that are important for insect growth. The last major group of insecticides inhibits components of insect mitochondria via disruption of the proton gradient, or inhibition of mitochondrial ATP synthase, or inhibition of electron transport individual mitochondrial complexes I through IV.

Insects have developed various strategies to adapt to toxic environment. There are several similar definitions of insecticide resistance coined by different agencies or individuals. The one I chose to present here is from the IRAC because it is simple and practical: insecticide resistance refers to 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species'(*1*). Resistance to insecticides is crucial for the survival of insect pest species. The specific molecular target or the site where

the toxin usually binds in the insect is referred to as a target site; examples are given in table 3.1. Because there are common target sites shared by more than one insecticide, cross-resistance occurs frequently. This means that in an insect population the heritable changes of the target site induced by selection pressure of one insecticide would render another insecticide ineffective even though the two might be structurally distinct but share a common target site. Discovery of new insecticide classes is thus highly desirable in order to remedy the cross-resistance problem. Another downside of traditional insecticides such as DDT or organophosphates is that many are effective but have high nonselective toxicity to humans and other animals. Finding eco-friendly insecticides will be a long and difficult process. In the past, stable synthetic insecticides became bio-accumulative globally (*2*). Scientists are continuously searching for new highly insect-specific compounds that are safe for long-term use. There is a coevolution arms race between thepest's development of insecticide resistance and our discovery of efficient new insecticides. Insects employ a number of general strategies to counter insecticides, including 1) reduced penetration 2) increased sequestration or excretion 3) behavior resistance 4) metabolic resistance 5) target-site insensitivity (Figure 3.1).

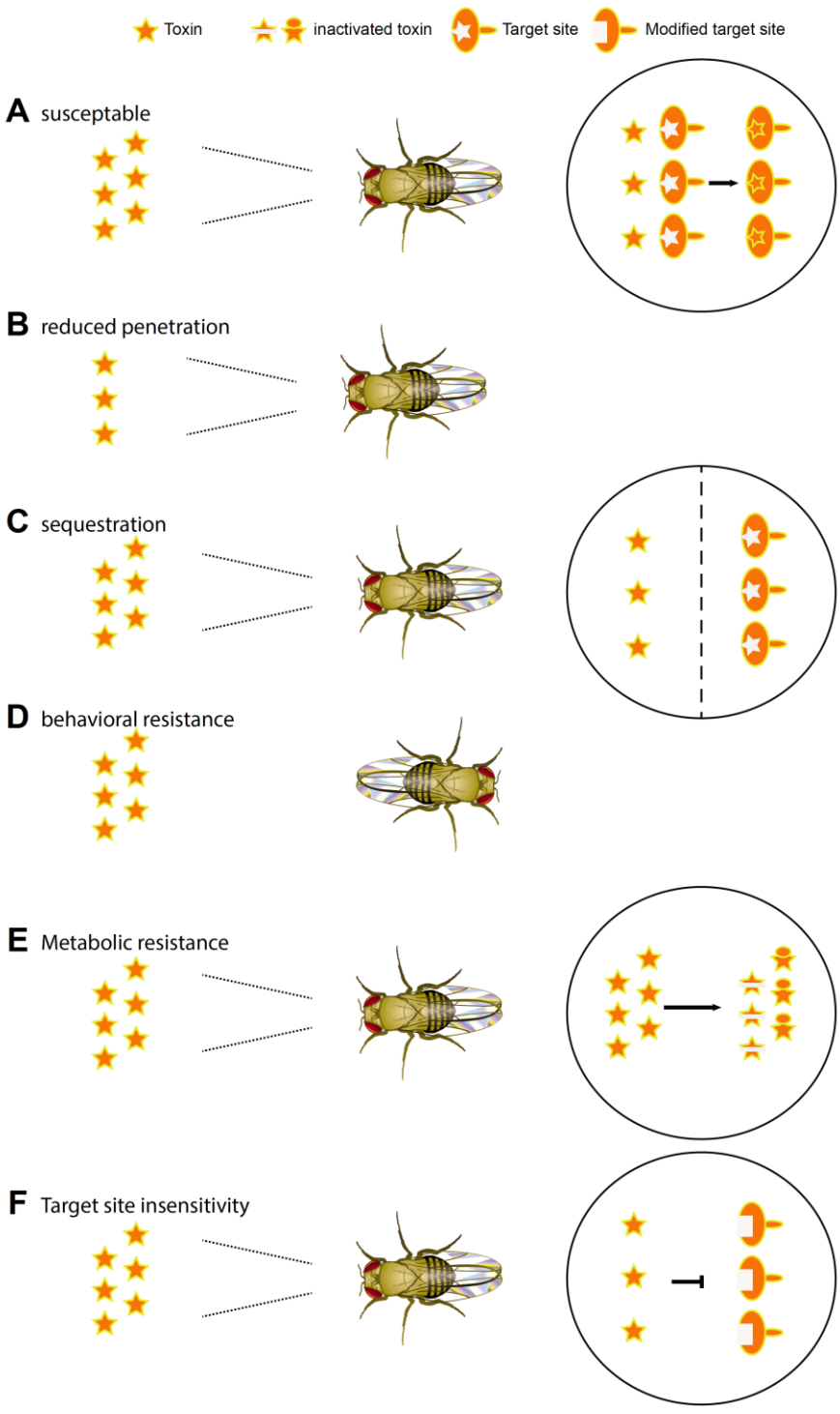


Figure 3.1 General mechanisms of insecticide resistance using *Drosophila melanogaster* as an insect model. (A). Insecticide function: a given insecticide taken up by the insect interacts with a molecular target site in a susceptible strain causes organismal death. (B). Reduced penetration: a heritable change in the insect population enables reduced penetration of insecticide or prevents it from entering the insect's body. (C). Sequestration: insecticide that entered the insect is bound by enzymes or proteins and transferred away from target sites to various detox tissues such as the fat body or hemolymph to safe guard. (D). Behavioral

resistance: a heritable mechanism/mechanisms in the insect population to avoid the toxic insecticide by changing their behaviors. (E). Metabolic resistance: detoxification enzymes in the insect degrade or add water-soluble polar side chains to the toxin, render the insecticide inactive and secreted out of the insect's body. (F). Target site insensitivity: a mutation changes the conformation of the target site without compromising too much of its function. As a result, the insecticide can no longer bind to the target site.

Reduced penetration:

Insects have developed mechanisms to prevent or reduce the entry/uptake of insecticides into their bodies. This phenomenon was first found in a resistant strain of house fly that had decreased penetration of DDT and dieldrin (*3*). It was later attributed to a gene named *pen* for penetration located on chromosome III (*4, 5*). Other insect examples of decreased penetrance were seen in permethrin-resistant flies and mosquito species *Culex pipiens* (*6-8*). It was proposed that reduced penetration could give detoxifying enzymes more time to metabolize or efflux and excrete the toxins before it reaches the target. In reality, resistance is often caused by a combination of decreased penetrance of insecticide and other resistance mechanisms such as target site insensitivity and increased enzymatic detoxification.

Increased sequestration or excretion

Once an insecticide enters the insect's body, it can be sequestered by enzymes or proteins and subsequently transferred away from its target site and stored in tissues such as the fat body or hemolymph to diminish its effect. This was thought to be the result of early interactions of insects with flowering plants that gives rise to the ability of insects to sequester toxic molecules from their plant based diet. Esterases and glutathione S-transferases usually mediate the sequestering process. Esterases can either confer broad-spectrum resistance by

rapid binding to the insecticide molecules so to increase the level of sequestration but not necessarily metabolism of the toxin, or can confer narrow-spectrum resistance by having a point mutation in its encoding gene that changes the substrate specificity so an entire class of novel insecticide with a common ester bond may be metabolized by the newly gained specificity of the esterase. Similarly, another mechanism exists to pump insecticide out before it reaches the target site, thus resulting in tolerance and resistance. ATP-binding Cassette transporters (ABC transporters) confers multidrug resistance by an efflux mechanism. ABC transporters are conserved from bacteria to humans arguing that it is the first line of defense against xenobiotics from the environment for all organisms.

Behavioral resistance

Toxins usually are ingested and the undesired taste is a hint to insects to stop feeding and turn away from harm. Any such avoidance behavior that increases the chance of survival of the insect and its offspring is termed behavioral resistance. There are numerous cases of *Drosophila* adult or larval aversion to chemical challenges in media as well as behavioral changes in oviposition in the females when media are supplemented with a drug. Such a behavioral change in larvae occurred in my own caffeine-based experiments. The avoidance of caffeine containing media was quantified by a dual-colour plate test where precisely half of the media plate consisted of normal medium colored with a blue dye and the other half was medium containing the drug of interest and colored red. The plated media offered a food choice by providing two adjacent

media types in two different colours for larvae to choose from. The avoidance behavior was measured by scoring the blue/red or purple colour in the abdomina of 50 larvae per plate on eight plates. Wild type larvae avoided red caffeine media 100%, with some red colored food in latter parts of their guts signifying the "taste-then-avoid" behavior. My interpretation of this is that the wild type larvae were able to taste the bitterness of caffeine in the red-coloured medium after initial ingestion and moved away. Thus, the red media was only seen in the lower abdomina. Other insect species such as the Diamondback moth or the German cockroach have such avoidance behavior as well (*9, 10*).

Target site insensitivity/ cross-resistance

Insecticide targets are mostly evolutionarily conserved. Insecticides therefore can target their mammalian counterparts; result in non-specific toxicity to other unintended mammalian targets including humans. Thus, it is of utter importance that we understand insecticide target sites and look for alternatives that bind to insect specific targets. Under evolutionary selection pressure, insects have evolved super races that are resistant to insecticides. Resistance in these cases was achieved when a pre-existing missense mutation in the insecticide target was steadily inherited in the population. Such mutations change insecticide targets to a conformation that decreases their affinity towards the toxin, but retains the normal function of the target or destabilizes its toxin bound conformation thus rendering the target insensitive to the insecticide. Compounds from the same chemical family usually share a common target site within the insect. However, structurally different compounds can inhibit the same target. Mutations in one

target can thus confer cross-resistance to various insecticides that share the common target. The IRAC recommends a rotation of insecticides that interact with different targets for effective pest control.

Metabolic resistance and transcription factors in metabolic insecticide resistance

Metabolic resistance refers to a situation where insects increase the rate of metabolism of the pesticide by overexpressing genes that encode detoxification enzymes or mutations that allow the detoxification enzyme to work more effectively leading to broad-spectrum resistance. Metabolic resistance is a widespread phenomenon found in a range of insect phyla including lepidoptera, coleoptera and diptera (*11-17*). This common mechanism by which insects evolve resistance has been extensively studied, and the corresponding literature on this topic is vast, for reviews see (*18-23*). Here, I will limit examples for *Drosophila melanogaster* to illustrate metabolic resistance mechanisms when appropriate. Most studies to date have elucidated the roles of cytochrome P450s, glutathione-S-transferases, UDP-glucuronosyltransferases, esterases and ABC transporters. In fact, a change in gene regulation is the underlying reason for the insecticide resistance phenotype, resulting in an increase in efficiency in one or more physiological aspects mediated by these enzymes: oxidation, conjugation to hydrophilic compounds and excretion.

Cytochrome P450s (CYPs or P450s) are a class of heme-thiolated monooxygenases that catalyze the oxidation of a wide range of compounds, including endogenous compounds such as steroid hormones, as well as exogenous toxins. P450s metabolize insecticides by N-, O- and S-alkyl hydroxylation,

aromatic hydroxylation, aliphatic hydroxylation and epoxidation, ester oxidation, thioether and nitrogen oxidation (*24*). In *Drosophila melanogaster,* P450s contribute to the resistance of 1,1,1-trichloro-2, 2-bis(4-chlorophenyl)ethane (DDT), a classic insecticide that was widely used in the 70s and a range of more recently used/developed insecticides such as neonicotinoids and pyrotheroid. These include, but are not limited to: CYP6G1, CYP6A2, CYP12D1, CYP6A8, CYP12A4, and CYP6W1 (*25-31*).

The molecular mechanisms by which P450-mediated insecticide resistance is conferred have been dissected to gain insights into mechanisms of resistance and cross-resistance, here using DDT resistance as an example. Studies in DDT resistance showed two types of inheritance of insecticide resistance: polygenic vs. monogenic (*32-34*). The relative importance of single gene vs. multiple genes in insecticide resistance is under very lively debate (*35*). Laboratory strains of *D.melanogaster* exposed to prolonged artificial DDT selection for 25 years are highly resistant to DDT. This DDT resistance is associated with all three major chromosomes. The lab strain's resistance is probably due to selection of a large number of factors of lesser effects (*36*) because in at least some species, the upregulation of a single P450 gene, *Cyp6g1,* is sufficient for DDT resistance (*25*). The reason for this phenomenon could be that lab-based populations are too small to include the extremely rare resistant variant. Analysis of detoxification microarray experiments with all the known P450 genes in *Drosophila* showed that different lab selection processes yielded different P450s for resistance (*37*). Moreover, overexpression of P450 genes with Gal4:UAS gene switches (*38*) in

the Malpighian tubules and fat body of transgenic fly showed that more than one P450 gene can confer DDT resistance (e.g. *Cyp12d1*) (*39*). However, resistant *Drosophila* strains collected from the field showed a single gene *Cyp6g1* over-transcribed in the DDT-resistant flies. The matter of polygenic vs. monogenic resistance is further complicated by the fact that the well-studied DDT-resistant 91-R strain was lab-selected and its resistance to insecticides is polygenic in nature. However 91-R contains the common DDT-Resistant allele (of *Cyp6g1*), which was presumably present in the starting population collected from the wild (*25*). In 2002, a comparison of 20 resistant and 20 susceptible *Drosophila* strains collected from 5 continents revealed that *Cyp6g1* is overexpressed in all 20 resistant strains (*25*). The overexpression of *Cyp6g1* was due to insertion of an *Accord* retrotransposon 291bp upstream of the *Cyp6g1* transcription start site. The regulatory elements present in the *Accord* long terminal repeat cause an increase in *Cyp6g1* expression in tissues important for detoxification (*40*). Sequencing of the *Accord* flanking region revealed that it was a single insertion event that resulted in a global expansion of resistance to DDT in *Drosophila melanogaster*. The role of overexpressed *Cyp6g1* in DDT resistance is supported by a striking parallel-evolved example in *Drosophila simulans*. An insertion of the transposable element *Doc* (an element different from *Accord*) into the equivalent position upstream of the *Cyp6g1* homolog in *Drosophila simulans* is also associated with increased *Cyp6g1* expression and DDT resistance (*41*).

The story of *Cyp6g1* does not end here. There are five known DDT-resistant alleles of *Cyp6g1* in *Drosophila melanogaster*, four of which were

caused by transposable elements inserted in the promoter region of *Cypg1*, and a single duplication event that formed two copies of *Cyp6g1* resistant allele in the same strain. The original resistant allele is referred to as the *A* allele because of the Accord retrotransposon insertion. The *A* allele underwent a duplication event to produce two copies of inserted *Cyp6g1*, each has one *Accord* insertion, in the same strain (named *AA* allele). The *AA* allele is further mutated by either insertion of a HMS-Beagle element in one of the Accord element to form the Beagle-Accord (*BA*) allele or a double replacement of Accord elements by a HMS-Beagle and a P-element (termed the *BP* allele). Finally, the terminal repeats of the P-element are scrambled in *BP* to give the *BP*Δ allele. All six alleles of *Cyp6g1* including the susceptible *M* allele exist in the wild. The multiple mutational steps of the *A* allele appears to be selected for higher resistance efficiency, the most susceptible to most resistant being *M*<<*AA*<<*BA*<<*BP* (*42*). These observations show that DDT resistance is adaptive; the more derived the allele the greater the DDT resistance. A first "pioneer" mutation with relatively low fitness value with respect to toxin resistance is often replaced by more robust higher fitness "settler" mutations in toxin resistance development as suggested by Taylor and Feyereisen (*43*).

The fact that there are multiple alleles of DDT resistance serves as a warning that the continued usage of large quantities of insecticides might select for stronger resistance mutations that lessen its effectivity over time. Likewise, increasing the dosage to compensate for this is detrimental to the environment and human health. The intensity of selection should be controlled by rotating different

insecticides that act on distinct physiological targets and by reducing the frequency and intensity of application. Higher levels of *Cyp6g1* expression correlate with a higher DDT-resistant phenotype, because *Cyp6g1* can actually metabolize DDT to its derivative DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) (*44*). *Cyp6a2* is another P450 enzyme that is associated with DDT resistance and metabolism (*26*).

Another enzyme associated with DDTase activity is glutathione-S-transferase (GST). GSTs are a family of conjugative phase II metabolic isoenzymes that catalyze the conjugation of glutathione substrate to xenobiotics to increase their solubility. GSTs also have glutathione-dependent peroxidase activities against endogenous lipid hydroperoxide and hydrogen peroxides. GSTs are categorized into several classes (ζ, θ, σ and ω). Most GSTs have a broad spectrum of substrate specificity on xenobiotic detoxification, however some of the members (specifically those of the MAPEG family) have very specific substrates (*45*). The best example of GST's detoxification function is a GST that is associated with DDT resistance; although what insecticide GSTs can detoxify is not limited to DDT. A GST can have more than one insecticide as its substrate (*46*). As early as 1953, an unidentified enzyme in houseflies was found with DDT dehydrochlorinase activity that converts DDT to the nontoxic derivative DDE (*47*). But not until 1984, was it discovered to be a GST (*48*). Since then GST activities have been associated with organochlorine, organophosphorus, and pyrethroid resistance (*22, 35, 49*). It is thought that there are two ways by which GSTs contribute to insecticide resistance, one is direct binding and sequestering

the insecticide, and the other is protection against oxidative stress generated as a by-product of insecticide metabolism. In *Drosophila melanogaster*, the microsomal GST containing fraction is associated with protection against oxidative stress that shortens the life span (*50*). This role of GST is also supported by the finding in the rice brown planthopper, *Nilaparvata lugens*, GSTs protects against oxidative damage created as a by-product of the insecticide pyrethroids toxicity (*51*). The PSU-R strain of *Drosophila melanogaster* is highly resistant to DDT due to overexpression of *GSTD1,* which has a DDT dehydrochlorinase activity that converts DDT to nontoxic DDE (*49*). This is consistent with the finding that overexpression of the *GSTD1* housefly homolog is associated with DDT resistance (*52*). Also, in the mosquito species *Anopheles gambiae* and *Aedes aegypti*, DDT resistance is associated with overexpression of delta or epsilon classes of GSTs, such as *GSTE2 (53, 54)*. The extent to which *GSTD1* contributes to DDT resistance in *Drosophila melanogaster* is unclear, especially when compared with *Cyp6g1* or *Cyp6a2*. Genomic tools such as RNA-Seq or microarray analysis could be used to compare global expression changes in isogenic susceptible strains vs. resistant overexpression strains. This would show relative expression of each enzyme involved in DDT metabolism and ultimately identifiy the factors contributing to DDT resistance.

UDP-glucosyltransferases are another substantial class of conjugative drug-metabolizing enzymes that eliminate various endogenous and exogenous compounds by catalyzing reactions where a glucosyl group is added to a lipophilic substrate. Much like GSTs, UGTs are found from bacteria to humans,

showing a fundamental role of their function in evolution. UGTs in humans are capable of metabolizing therapeutics, dietary chemicals, environmental pollutants as well as endogenous compounds such as bilirubin, hydroxysteroids, thyroid hormones, neurotransmitters, fatty acids and eicosanoids (*55*). In *Drosophila melanogaster*, several UGTs are induced by phenobarbital, a strong inducer of detoxification responses (*56*). GSTs and UGTs are conjugative Phase II detoxification enzymes.

Esterases are enzymes that 1) directly catalyze hydrolysis of drugs and insecticides to inactive metabolites by a common ester bond or 2) sequester insecticides resulting in broad-spectrum resistance. Point mutations in carboxylesterase-encoding genes reduce the ability to hydrolyze the carboxylesterse substrates and increase hydrolysis of organophosphorous (OP) in the OP-resistant houseflies (*57*). The equivalent point mutation resulted in OP-resistance in Australian sheep blowfly *Lucilia cuprina* and for mosquito the OP-resistant acetylcholinesterase-1 (*58, 59*). Site-directed mutagenesis of human butyrylcholinesterase to alter the equivalent amino acid of the insect active site also gave rise to a novel OP hydrolysis activity (*60*). Interestingly in *Drosophila*, the ortholog of the Australian sheep blowfly *Lucilia cuprina* gene *LcaE7* called *EST23* is a part of a large ester gene cluster; the corresponding amino acid change was not associated with OP-resistance (*61*). Besides point mutations in esterase genes, overexpression of esterases by either upregulation or gene amplification can lead to the emergence of insecticide resistance as well. The best examples are in the mosquito *Culex* species (*62*). Gene amplification of OP-inactivating

carboxtlesterases in *C. pipiens* is associated with sequestration but not necessarily the breakdown of insecticides (organophosphorous and carbamates) (*63*). Amplification of one or more esterase loci, sometimes in combination, is responsible for the observed OP-resistance. The same is true for other *Culex* species. In some *Culex* species, this energetically costly overexpression of carboyxlesterase is further replaced by mutation and gene duplication of the direct insecticide target acetylcolinesterase (*64*). This is an example of target site insensitivity rather than sequestration in the emergence of insecticide resistant strategies. In the peach potato aphid, *Myzus persicae*, resistance to OP is associated with either amplification of *esterase-4* (*E4*) or an alternative truncated form, *'fast'-E4* (*FE4*) up to a remarkable number of 80 copies in its genome. This results in esterase protein making up to 1%-3% (depending on the study) of the total body weight (*65*). Intriguingly, the metabolism of insecticide itself is slow, but the E4 esterase acts as a large sponge to sequester the insecticide. Gene amplification is not unique to esterases; P450 and an ABC transporter genes are found amplified in the dengue vector mosquito *Aedes aegypti* and aphid *Myzus persicae* (*23, 61, 66*).

The ABC (ATP-binding cassette) transporter protein family is the largest efflux pump family with subfamilies ABCA to ABCG found in all kingdoms of life. They require binding and hydrolysis of ATP to transport substances across the lipid membrane. Human ABC transporters are very well studied for their function in absorption, distribution and excretion of drugs and other therapeutic agents. ABC transporters are associated with multiple drug resistance phenotypes

in vertebrates and invertebrates alike, the best-studied invertebrates being nematodes. Fewer arthropod ABC transporter genes have been reported, however the interest of studying arthropod ABC transporter-mediated insecticide resistance has been steadily growing in the recent years (reviewed in *67*). In insects, ABC transporters are implicated in transport of and/or resistance to 27 insecticides and acaricides (poisons against tick and mites) including carbamates, macrocyclic lactones, organophosphous, neonicotinoids, pyrethroids, cyclodienes, benzoylureas, phenylpyrazoles, and DDT, belonging to 9 structurally distinct chemical classes and several modes of action (reviewed in *68*). Here I describe reported cases of ABC transporter mediated resistance to three commonly used insecticides. 1) *ABCB FT/p-gps* was found to be highly expressed in the cuticle of tobacco budworm *Heliothis virescens* larvae and was further overexpressed in carbamate resistant strain. Using the ABC transporter inhibitor quinidine greatly decreased the LD50 of a resistant strain to carbamate and led to a two to three fold accumulation of C^{14}-labelled carbamate in the uninhibited control (*69, 70*). Other studies have shown that related ABC transporters were upregulated in green peach aphid *Myzus persicae* exposed to pirimicarb (*71*). 2) The same *ABCB FT/p-gps* was associated with efflux of OPs. In the tobacco budworm *Helicoverpa armigera*, different OPs stimulated ATPase activity of purified ABCB FT/p-gps. *ABCB FT/p-gps* is also upregulated in temephos (another OP) treated mosquito *Aedes aegypti* larvae. Temephos in combination with an ABC transporter inhibitor verapamil increased the toxicity by 57% (*72, 73*). 3) An ABCG is over-transcribed in DDT-resistant *Drosophila melanogaster* and *Anopheles arabiensis*

strains (*74, 75*). In the DDT-resistant *D. melanogaster* 91-R strain, excretion of DDT and its metabolites is five fold higher compared to the susceptible control strain (*76*). The ABC transporter inhibitor verapamil reduced the LC50 of 91-R by ten fold but did not affect the susceptible strain. RNAi knockdown of two ABCB **(**mdr50 and mdr65**)** and one ABCC (dMRP/CG6214) transporter gene(s) that were overexpressed (1.3-fold) in resistant flies increased their susceptibility to DDT. ABC transporters are therefore most likely associated with efflux of DDT in the *D. melanogaster* 91-R strain (*76-78*). The study of ABC transporters with respect to other types of insecticides is scarce, however, recent advances in genomics and the usage of insect models other than *Drosophila melanogaster* has opened up new possibilities. Microarray gene expression, RNA-Seq and qPCR studies revealed that ABC transporters were upregulated in association with pyrethroid resistance in three different insect species (*79-81*). Pyrethroid stimulation of ATP hydrolysis and verapamil synergistic toxicity has been reported in several insect species. An ABC gene was identified by microarray analysis, because it was overtranscribed in adult *Bemisia tabaci* flies of a thiamethoxam-resistant strain (*82, 83*). Verapamil has been shown to enhance toxicity of three neonicotinoids in the honeybee (*84*). Nevertheless there is no direct evidence that links ABC transporter function to neonicotinoids resistance.

In summary, arthropods have developed a variety of strategies to deal with environmental toxins. The coping mechanisms are divided into two classes: pharmacodynamics or reduced response to toxins and pharmacokinetic or reduced exposure to toxins. The decreased response is achieved by mutations that disrupt

the interaction of insecticides with its cellular targets also referred to as target site insensitivity. Mechanisms for decreased exposure includes behavior resistance, reduced penetration, sequestration, altered metabolism and excretion of insecticides. In most cases, point mutations that render target sites insensitive or detoxification by altered metabolism/sequestration led to an insecticide-resistant phenotype. The detoxification process is divided into three phases. In phase I, cytochrome P450 monooxygenases and carboxylesterases make toxins more reactive and water soluble by adding hydroxyl, carboxyl and amino groups which is often followed by conjugation of polar side chains mediated by the phase II enzymes glutathione-S-transferases and UDP-glycosyltransferase. Finally, in phase III the polar hydrophilic compound or conjugates are pumped out of the cell by ABC transporters. Occasionally, ABC transporters can directly transport the unmodified toxin out of the cell efficiently. This sometimes is referred to as phase 0 detoxification (*67*). A word of caution, terms of "phase I", "phase II" and "phase III" reactions were used traditionally in characterizing enzymes in xenobiotic metabolism, however mechanistically unrelated processes such as GST or UGT activities are grouped together and a sequential nature is implied, but does not always exist (*85*).

The polygenic nature of metabolic insecticide resistance has led the scientists in the field to hypothesize that there is a hitherto unknown trans-regulatory factor network that controls the expression of these detoxification enzymes (*86-88*). Since our intial understanding of transcription regulation was via the discovery of repressors in bacteria, it was hypothesized that in susceptible

insects the repression of detoxification enzymes were represented. A mutation in the repressor no longer allows it to reduce expression levels of P450 and other enzymes in the resistant strain. However, it is known that constitutive up-regulation of detoxifying enzymes by transcription activation is most common, and gene amplification has been seen in some cases. In order to mount a quick and effective defense against harmful chemicals coordinating numerous drug-metabolizing enzymes, detoxification of xenobiotics is likely orchestrated by key transcription factors. In fruit flies, *Drosophila hormone receptor 96* (*DHR96*) and *Cap-and-collar isoform-C* (*CncC*) (the *D. melanogaster* homolog of human *Nrf2*) are transcription factors known to control the expression of many detoxification genes (*89*). However, the majority of the PB induced detox genes in a recent study were DHR96 independent (*56*). Likewise, in the DDT resistant 91-R strain, approximately 20% of the genes differentially expressed are known CncC target genes. Thus, the CncC pathway is only partially responsible for the over transcription of detoxification enzymes seen in 91-R (*90*). This raises an interesting possibility that one or more additional transcriptional regulators fulfill the role of xenobiotic detoxification regulation in *D. melanogaster. Drosophila* is an excellent genetic model to dissect the regulation of detoxification and specific mechanisms of insecticide resistance. Elucidation of such factors will aid our understanding of how insects regulate metabolic resistance to environmental toxins. This may allow better pest control by synergistically inhibiting detoxification regulators in the presence of insecticides.

3.1.2 "omics" approaches used for studying insecticide resistance

Omics approaches comprise large-scale genome-wide or proteome-wide surveillance of changes in an integrated manner that allows us to gain a systemic understanding of how insects respond to and become resistant to natural or synthetic toxins. Comparisons are routinely carried out with susceptible strains vs. resistant strains or unchallenged vs. challenged organisms. Several drugs are used for this type of study. The vast array of omics technologies available, especially the recently developed Next-generation sequencing, can comprehensively describe virtually all components and processes in an organism. The challenge becomes how to make sense of such a wealth of information and to extract what is useful.

3.1.3 *Drosophila* as a model to study insecticide resistance

Because of gene conservation amongst insects, *Drosophila melanogaster* is used as a genetic model to study insecticide resistance in pests. Some *Drosophila* species such as *Drosophila melanogaster* are not considered pests. However, through enough incidental exposure to insecticides in the environment over the years some strains have developed resistance to older insecticides. In the laboratory, repeated selection by insecticide exposure and mutagenesis strategies has provided the opportunity for resistance to arise. Other species are of agricultural concerns. Spotted wing *Drosophila*, *Drosophila suzukii* is a serious pest of soft fruits and berries in the wild (*91*). Since its identification in 2009, *D. suzukii* continues to spread and it is now widely distributed in Asia, North

America and Europe. In British Columbia, Canada, *Drosophila suzukii* is known to infest wild and cultivated raspberry, blackberry, blueberry, strawberry, cherry, peach, nectarine, apricot and plum, among other fruits and suspected in hardy kiwifruit (*92*). Understanding insecticide resistance in *Drosophila* model will greatly facilitate *Drosophila suzukii* pest control.

Significance: While it is widely accepted that multiple mechanisms contribute to insecticide resistance in a single insect genome, the relative importance of structural (target site insensitivity) vs regulatory (transcriptional) mutation is still under considerable debate. In the past 100 years, our options of pest control were severely limited by our lack of understanding of the relevant biochemical and molecular aspects of insect physiology. One of the biggest problems of using pesticides is that the pesticides have side effects in vertebrates because these insecticides have evolutionarily conserved targets. Also the earliest generation of insecticides tends to be very stable in the environment. As a result they enter tissues of animals and plants (where they often accumulate), where they have been found in the germ line. They are also passed on in the food chain, and accumulate in the biosphere. Such bioaccumulation creates a huge negative impact on ecosystems, as described in Rachel Carson's famous book Silent Spring (*85*). Obviously, our efforts need to concentrate on development of insect-specific control methods that have minimal impact on vertebrates and other organisms. For this purpose, understanding of insecticide response and resistance at the molecular, cellular and organismal level gives us tremendous opportunities to develop novel pest control strategies that are of immense practical value. In

particular a better understanding of the transcriptional regulation of xenobiotic detoxification is crucial to aiding us in safe and effective pest control.

Focus of this study: Determining the transcriptional response to caffeine in wild type *Drosophila melanogaster* larvae will help to characterize and elucidate the molecular mechanisms insects employ to respond to xenobiotics.

3.2 Materials and Methods

Making plates and fly media

Grape juice agar plates

30-35g of Agar (Select Agar®, Powder (Invitrogen™) Catalog Number 30391-023) was added to 750 ml ddH_2O and heated until agar dissolved completely. 250 ml Welch's grape juice was added to the mixture and mixed well. 0.5 g of antifungal agent methylparaben dissolved in 20 ml of 95% ethanol was then added to the mixture right before pouring. This mixture makes 150 to 200 small 35 mm plates. Store plates at 4ºC. Recipe can be scaled down to make smaller batches of grape juice agar plates.

Yeast plates

Supermarket variety of dry baker's yeast was added to ddH_2O in a 50ml beaker, mix until smooth and moist but not runny. To encourage egg deposition, ddH_2O can be substitute by a mix of 1 part cider vinegar 2 parts ddH_2O for optimal results. Cover beaker with parafilm (PARAFILM®M P7793-1EA Sigma) and store at 4ºC. To make yeast plates for staging experiments, wet round filters were inserted and layered so that two white #1 were on the bottom and one black #551

on top in 90 mm petri dish covers (the top) to allow spotting the whitish larvae more easily. Perforated petri dish bottoms were used as covers. Individual plate was sealed with parafilm. Moisture was checked daily and more water added if plates appeared dry.

Drug supplemented media

For making caffeine food, 200 mM caffeine (Sigma) stock solution was heated in a 50ºC water bath to dissolved caffeine crystals with occasional shaking. Caffeine solution was added to hot cornmeal-yeast-molasses media to a desired final concentration in blender, blending on high for 3 min. Caffeine-containing medium was poured into plates or vials. Bromophenol blue or carmine red food dye was added to media to track food consumption or distinguish different types of media, standard vs caffeine.

Starvation treatment

20% sucrose stock solution was filter-sterilized and frozen for long-term storage. Small 35mm petri dishes were lined with cutouts of black filter paper and a small ball of black filter paper was put in the center to mimic a blob of yeast paste. 2% sucrose solution was added to the plate. Newly molted L3 larvae were washed with 1X PBS in glass depression plates before transferred to starvation plates. Liquid nitrogen was used to flash-freeze 7-10 larvae per sample collected at the end of 4 hr.

20-hydroxyecdysone (20E) rescue

To make 10 mg/ml 20E stock, dissolve 100 mg of 20E powder (Steraloids.Inc catalogue ID C3020-000) into 10 ml 95% ethanol. 50 µl of 20E stock was added

to 1.5 ml of hot cornmeal-yeast-molasses media, blending for 3 min. This gives a final concentration of 0.33 mg/ml of 20E and approximately 3% ethanol. For control food, 50 µl of 95% ethanol was used rather than 20E stock. Larvae that had ecdysed within 1 hr of each other were added to freshly made plates in groups of 50 animals, and allowed to feed at will. Rescue was scored as number of pupa /50 larvae.

Third instar larvae staging and sample collection

Parental stocks were expanded in bottles to achieve the optimal population density to ensure healthy offspring. 2-3 day old adults collected from such bottles were allowed to mate for a day in plastic egg cages with 1-2 changes of grape juice plates with a blob of yeast paste. This helps flies to get used to an environment with frequent disturbance. On the second or third day, fresh room temperature (RT) grape juice plates were used in the cages as cold plates tend to shock flies and subsequent egg lay could be poor. A pre-lay for 2 hr to rid the eggs held in the females from the night before was used. A repeated pre-lay for another hour was carried out if necessary. Subsequent embryos collected after 1-hour egg-lays were transferred onto yeast plates. Emerging larvae were staged at the L2 to L3 molt in half hour intervals. PBS-rinsed newly molted L3 were transferred to corn-molasses-yeast media or corn-molasses-yeast media supplemented with 8 mM caffeine. After 4 hr, 7-10 larvae were collected as one sample and flash-frozen in liquid nitrogen.

RNA preparation and cDNA synthesis

Total RNA extraction from whole larvae or adults

Total RNA was extracted following a modified TRIzol (Invitrogen) protocol: Plastic pestles were used, using a motorized homogenizer, to crush samples that were frozen in -80 ºC or liquid nitrogen to a fine powder, in the presence of 400 µl TRIzol (Roche). Care was taken so that the total volume of the sample would not exceed 10% of the volume of TRIzol in the homogenization step, otherwise DNA contamination could result. The volume of TRIzol was then filled to 1 ml and mixed by vortexing, followed by a 5 min incubation at RT. 200 µl of chloroform was added to the tube. The mixture was shaken vigorously by hand for 15 second, and incubated at RT for 3 min. Samples were centrifuged for 15 min at 4 ºC and 12000 g. The upper, colorless aqueous phase was then transferred to a fresh tube containing 500 µl isopropanol. The mixture was mixed by inversions and incubated at RT for 10 min before centrifuged for 10 min at 4 ºC and 12000 g. Supernatant was removed. The resulting pellet was washed gently with 75% ethanol then centrifuged at 4 ºC 7500 g for 5 min. Supernatant was poured out and the last drops of liquid on the wall of tube were pipetted out. The pellet was air dried at RT for 10 min till completely dry then was dissolved in 100 µl Nuclease-free water (Life technologies Ambion® Catalog NumberAM9930). 200 µl of chloroform was added to the tube. The mixture was shaken vigorously by hand for 15 sec, and incubated at RT for 3 min. Samples were centrifuged for 15 min at 4 ºC and 12000 g. The upper aqueous phase is added to a fresh tube containing 10 µl of 8M RNase-free LiCl solution and 275 µl of 100% technical

grade ethanol. Mixture was mixed by inversion and incubated in an ice-water bath for >2 minutes or O/N at -20ºC freezer. Mixture was centrifuged for 30 min at 0 ºC and 12000 g. Supernatant was poured out and the pellet was gently washed with 1 ml of 75% ethanol then centrifuged for 2 min at 4ºC and max speed. Supernatant was poured out and the last drops of liquid on the wall of tube were pipetted out. The pellet was air dried at RT for 10 min until completely dry then was dissolved in 100 µl Nuclease-free water. Repeat the following steps once again: 200 µl of chloroform was added to the tube. The mixture was shaken vigorously by hand for 15 sec, and incubated at RT for 3 min. Samples were centrifuged for 15 min at 4 ºC and 12000 g. The upper aqueous phase is added to a fresh tube containing 10 µl of 8M RNase-free LiCl solution and 275 µl of 100% technical grade ethanol. Mixture was mixed by inversion and incubated in an ice-water bath for >2 min or O/N at -20ºC freezer. Mixture was centrifuged for 30 min at 0 ºC and 12000 g. Supernatant was poured out and the pellet was gently washed with 1 ml of 75% ethanol then centrifuged for 2 min at 4ºC and max speed. Supernatant was poured out and the last drops of liquid on the wall of tube were pipetted out. The pellet was air dried at RT for 10 min until completely dry then was dissolved in 10 µl of nuclease-free water at RT by finger flicking or 4ºC O/N. Solution should be colorless but slightly viscous.

RNA normalization

The concentration of 1/10-diluted RNA was measured in duplicates with NanoDrop (Thermo Scientific); the mean value was used for normalization calculation. All original RNA samples were normalized to 1 µg/µl. Sub 1 µg/µl,

concentrations were normalized to the next possible round number for ease of pipetting 1 µg for cDNA synthesis. Common numbers were 500 ng/µl or 250 ng/µl. RNA were aliquoted as 5 µl aliquots, flash frozen in liquid nitrogen and stored at -80 ºC for long term storage.

RNA integrity was assessed by analyzing 1/10 RNA dilution with Agilent 2100 Bioanalyzer system. The Agilent RNA 6000 Nano Kit was used for this purpose (Protocol(*2*)).

cDNA synthesis

Each 20 µl reaction was composed of 1 µl of the normalized RNA (1 µg/µl) in 9µl of nuclease free water, and 10 µl of 2x master mix of High-Capacity cDNA Reverse Transcription Kit (Life technologies Invitrogen™ Catalog Number 4368814). 2X mastermix was made according to the following table.

Component	Volume/Reaction (µl)
10X RT Buffer	2 µl
25XdNTP mix (100 mM)	0.8 µl
10X RT Random Primers	2 µl
Multiscribe™ Reverse Transcriptase	1 µl
Total	10 µl

The reverse transcription reaction was carried out in the following thermal cycler conditions:

	Step 1	Step 2	Step 3	Step 4
Temperature (ºC)	25	37	85	4

Time	10 min	120 min	5 min	∞

Resulting cDNA (first strand) was stored at 4ºC overnight or -20ºC for long-term storage.

Quantitative real-time PCR (qPCR)

Primer validation

To establish the dynamic range and amplification efficiency of new primers, primers were validated prior to being used in a gene expression study. To do so, first a 3.2 µM working concentration stock of primer mixes was made by adding 8µl of each 100 mM primer stock into 484 µl of nuclease-free water. A serial dilution of cDNA with a dilution factor of 1/4 resulting concentrations 1/4, 1/16, 1/64, 1/256, 1/1024 was carried out with a cDNA sample where the gene of interest is expressed. Gene of interest was compared to an endogenous control gene *ribosomal protein 49* (*rp49*).

Individual SYBR mastermix was made for each gene tested with 5µl SYBR and 2.5 µl of 3.2 µM primer mix. Each dilution runs in duplicates on qPCR plate. 7.5 µl of matermix and 2.5 µl of the appropriate cDNA dilution were in each qPCR reaction. Applied Biosystems® StepOnePlus™ Real-Time PCR system was used with the following thermocycling parameters:

	Step 1	Step 2*	Step 3*	Step 4
Temperature (ºC)	95	95	60	Melt curve stage
Time	2 min	2 sec	20 sec	1 hour

* Step 2 and 3 were repeated consecutively 40 times

The qPCR machine (StepOnePlus, Applied Biosystems) was run in standard curve mode, not comparative CT mode. Standard curves needed to be set up for each primer pair including *rp49*

Efficiency of amplification was determined by comparing the standard curves of genes of interest to that of *rp49*. These curves should be parallel to each other. Specificity of the primer pairs was determined by melt curve analysis of the end products. A single peak in the melt curve showed there was no secondary product.

ΔΔCT determination of gene expression

Plate set-up

qPCR was performed on 3 or 4 biological samples each tested in triplicate (in total, 9 to 12 replicates of the same gene in one condition). Each cDNA sample representing one biological sample was diluted 1/20 then 2.5 µl (equivalent of 6.25 ng) of synthesized cDNA gone into each qPCR reaction. Master mixes were made of 5 µl SYBR (ABI Power SYBR® Green PCR Mater Mix, later on D-Mark Biosciences KAPA SYBR® Fast Master Mix (2X) ABI PrismTM) and 2.5µl of 3.2µM primer mix for each gene tested and that of endogenous control *rp49*. The Applied Biosystems® StepOnePlus™ Real-Time PCR system was used in comparative CT mode with the following thermocycling parameters (example protocol for D-Mark Biosciences KAPA SYBR® Fast Master Mix (2X) ABI PrismTM):

	Step 1	Step 2*	Step 3*
Temperature (ºC)	95	95	60
Time	2 min	2 sec	20 sec

* Step 2 and 3 were repeated consecutively 40 times

qPCR Analysis

Comparative CT method was used in analysis. In principal, the expression difference between the gene of interest (GOI) and the endogenous control (in most instance, *rp49* gene) in a particular sample was calculated as the ΔCT. Then the difference of ΔCT's between different samples was calculated as the ΔΔCT. The linear fold change is shown as 2ˆ-ΔΔCT.

Affymetrix GeneChip *Drosophila* genome 2.0 microarray

See (*93*) for detailed notes, volume of buffers were used according to an older version of the manual (2009). Sequences used in the design of the GeneChip *Drosophila* Genome 2.0 Array were selected from Flybase version 3.1

Day one: A serial dilution of poly-A RNA control stock with poly-A dilution buffer was made 1/500000. 2 µl of diluted poly-A RNA, 4 µl first-strand buffer mix, 1 µl first-strand enzyme mix and 300 ng of total RNA in a tube was incubate using a PCR thermocycler at 42ºC for 2 hr. 5 µl second-strand buffer mix, 2 µl Second-strand enzyme mix and 13 µl ultra-pure water was added to the tube and incubated at 16ºC for 1 hr, then 65ºC for 10 min. 4 µl IVT biotin label, 20 µl IVT labeling buffer and 6 µl IVT enzyme mix at RT were added to the tube then the labeling reaction was incubated O/N at 40ºC (16 hr).

Day two: 10 µl of homogenous bead solution and 50 µl of amplified RNA (aRNA) binding buffer concentrate were added to the tube from day one. The total volume in the tube should double.

Mixture was transferred to a 0.6 ml tube, 120 µl in total. 120 µl 100% ethanol was added. Mixture was gently shaken for 2-5 min at 400 RPM on Eppendorf MixMate®. Magnetic beads werecaptured for 5 min with a magnetic stand; supernatant was aspirated without disturbing the bead aggregate. 100 µl aRNA wash solution (alcohol added) was added to the tube and shaken for 1 min at 700 rpm on MixMate. aRNA wash and bead capture steps were repeated twice. The beads were dried by shaking for 1 min at 1200 RPM to evaporate residual ethanol from beads. 50 µl pre-heated (50 ºC) aRNA elution solution was added to the tube and shaken for 3 min at 1200 rpm till beads were fully dispersed. Beads were captured for 5 min until the solution is clear. The supernatant, which contain the eluted aRNA, was transferred to a fresh tube. 2 µl of aRNA was taken to spec concentration by NanoDrop and 2 µl more was used for Bioanalyzer. Smaples were stored at -20ºC or used immediately in fragmentation step.

12 µg of aRNA, 6.4 µl of Array fragmentation buffer were added to nuclease-free water to a total volume of 32 µl. Mixture was incubated at 94ºC for 35 min. 2 µl was used for Bioanalyser. Fragmented aRNA samples were sent to microarray facility for hybridization and scanning.

Microarray analysis

The RMA software (*94*) was used to calculate expression values from the Affymetrix Raw files, and another software package, LIMMA (*95*), was used to determine fold-changes and statistical significance.

Data mining

Comparisons of various data sets were carried out using Microsoft® Access, in particular to determine common (=overlapping) genes in different gene sets. Cross comparisons of multiple data sets was performed by constructing pairwise comparisons first and then finding the overlapping gene set of the pairwise comparisons. GO term enrichment was annotated using GOstat by Tim Beißbarth(*96*). Statistical significance of the enrichment as well as the overlaps were determined using Excel using the Chi Square test.

Fluidigm 48.48 chip and 96.96- high throughput qPCR

Assay design:

Probe-based (Roche Locked Nucleic Acids, LNA) qPCR assays were designed using the web-based Probe Finder software from Roche Applied Science at: https://www.roche-applied-science.com/webapp/wcs/stores/servlet/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Library&langId=-1#tab-3

Drosophila melanogaster (fruit fly) was selected as target organism. Target gene was selected by entering the gene name. Primers targeting the desired transcript were designed automatically with "automatically select an intron spanning assay" selected on the bottom of the page. Both FlyBase curated collection that starts with FBtr or the NCBI reference sequence can be used.

Preparing Primer-Probe mixes and Pre-amplified samples:

100x mixture of the forward and reverse primers for each of the assays was prepared according to the following recipe:

100X Primer Pair Mix	Volume (µl)
Forward primer (100 µM)	20
Reverse primer (100 µM)	20
Nuclease-free water	60
Total	100

Probe-primer mixes were made by mixing 8 µl of 100x primer mix and 4µl of probe. Probe-primer mixes can be kept for at least a week, protected from light, at 4°C and used for several chips. Up to 37 GOI assays and 5 endogenous controls can be run on one 48.48 chip. Two probe-primer mixes were prepared for endogenous control gene rp49. Running each in duplicate on a 96 well format qPCR machine with TaqMan chemistry validated probe-primer mixes for new primer sets. In this step, a master mix was first made using the following recipe:

Ingredients	Volume (µl)
1/20 diluted cDNA sample	2.5/rxn
TaqMan Universal Master Mix	5/rxn
Nuclease-free water	2/rxn
Total	9.5/rxn

0.5 µl of Probe-primer mix was added per reaction.

A successful Probe-primer mix should produce an amplification curve with an "S" shape. Probe-primer mix with a flat curve would fail on Fluidigm chips and were therefore replaced by a new primer pair.

A 4x multiplex primer mix for the Pre-Amplification reaction was made by pooling 100X primer mixes of each assay to be included in the chip or chip batch. The concentration of the 100X Primer Pair Mix is 40 μM (or 20 μM per primer), the final concentration of each primer in the 4X multiplex primer mix is 400 nM

Components	Volume (μl)
Assay 1	1
Assay 2	1
Assay 3	1
Etc…Up to 100 assays	1 each
Nuclease-free water	100 minus number of assays
Total	100

Assay= 100X primer pair mix

For pre-amplification reactions, the following master mix was made using ABI TaqMan PreAmp Master Mix (PN 4391128):

Components	Volume (μl)
4X multiplex primer mix	2.5/rxn
2X TaqMan PreAmp	5/rxn
Total	7.5/rxn

2.5 μl of 1/20 diluted cDNA was added.

Reactions were under the following thermal cycler conditions:

	Step 1	Step 2*	Step 3*
Temperature (ºC)	95	95	60
Time	10 min	15 sec	4 min

* Step 2 and 3 are repeated consecutively for 14 cycles

Pre-Amp reactions were tested by doing a pass/ fail test on a 96well format qPCR machine using a previously tested endogenous control, usually rp49.

The following master mix (for 16 pre-amp samples run in duplicate on qPCR) was used:

Components	Volume (µl)
Rp49 100X primer mix	8
Probe (#105)	4
Nuclease-free water	160
TaqMan Universal Master Mix	200
Reaction volume	9.5/rxn

0.5 µl of 1/5 diluted pre-amp samples were used per reaction. Passing samples should show mid-ranged CT values and S shaped amplification curves. Failed samples show low/no CT values and flat amplification curves, these samples often fail on the Fluidigm.

Loading and Running the Chip:

Preparing Assay Mixes:

The 10X assay mix contains Fluidigm, DA Assay Loading Reagent (PN 85000735) and Roche UPL Probes as the promer mix.

First, a master mix was prepared with DA Loading reagent and water using 160µl of DA loading reagent and 65µl of water. 4.5µl of this master mix was loaded into each of the 48 wells on the left half of a 96 well plate. Using a previously prepared map showing the location of each assay on the plate, 2µl of promer mix was pipetted into the same 48 wells.

Preparing Sample Mixes:

The following sample master mixes were prepared using ABI TaqMan Universal PCR Master Mix without UNG Erase (PN 4324018) and Fluidigm DA Sample Loading Reagent (PN 85000735):

Component	Volume (µl)
TaqMan Universal Master Mix	200
DA Sample Loading Reagent	20
Total	220

4µl of sample master mix was dispensed into each of well on the right side of the aforementioned 96-well plate. 2.5µl of Pre-amplified sample was added into the appropriate well according to a previously prepared map. The plate was sealed and stored shielded from light. The plate was vortexed with MixMate and spun down prior to loading the chip. To prime the chip with control line fluid, control line fluid was injected slowly through the openings on the side of the chip. Any drip on the array surface interferes with fluorescence detection in later steps thus needed to be avoided.

Running the Chip

5µl of the appropriate assay mix was loaded into the inlets on the left side of the primed chip. 5 µl of the appropriate sample mix was loaded into the inlets on the right side of the primed chip. Any air bubbles was removed promptly with a clean pipette tip. The IFC Controller MX was used to run the '113x Load mix' script to load the samples and assays into the chip. The software Biomark Data Collection Module was used to run the loaded chip in the Biomark instrument. Tamra was used as the fluorescent dye of the probes. The following thermal cycles in the 'April 09 file' on the Biomark computer was used

	Step 1	Step 2*	Step 3*
Temperature (ºC)	95	95	60
Time	10 min	15 sec	1 min

* Step 2 and 3 are repeated consecutively for 40 cycles

A run should be complete in ~2.5hr. Chip run data was stored on the desktop in the UofAlbertafolder according to the barcode number on the chip, unless otherwise specified.

***Drosophila* stocks and husbandry**:

All crosses were carried out at 25°C, and flies were maintained on media formulated at the Bloomington *Drosophila* Stock Center at Indiana University (BDSC) with *p*-Hydroxy-benzoic acid methyl ester or propionic acid as the fungicide. Stocks were obtained from the BDSC, the Vienna *Drosophila* RNAi Center (VDRC), or maintained/generated in our laboratories where specified. Fly stocks used were:

yw; Isogenized 3rd FRT82B

y1 w1118; P{Cbz}2/CyO (Bloomington 7221)

w1118 P{UAS-bsk.DN}2 (Bloomington 6409)

hs-Gal4

w118

Heat shock protocol:

2nd instars of the appropriate genotype were selected the day before (for *hs>cbz-DN*, non-GFP larvae were selected because *cbz-DN* was balanced over *CyO; Act5C-GFP*) and were allowed recovering on yeast plates over night. Late 2nd instars were transfer to fresh food vials and submerged in 38ºC water bath for 30 min. After 2 hours of recovery time, heat shocked larvae were transferred back to yeast plates for another hour. Newly molted 3rd instars were put onto media containing 0 mM or 8 mM of caffeine for 4 hr before collected for RNA extractions.

3.3 Results

3.3.1 Caffeine-induced transcriptome response in wild type early L3 *Drosophila* larvae

The transcriptome of *Drosophila melanogaster* 3rd instar larvae was monitored in response to the dietary administration of the xenobiotic compound, caffeine. Albeit not commonly used as an insecticide, as a natural pesticide caffeine has the ability to induce genes encoding detoxification enzymes including CYPs, GSTs, and UGTs in *Drosophila* and other insects (*30, 56, 89, 97-*

99). The dosage of caffeine is crucial for this drug study. I empirically determined the appropriate caffeine concentration that is high enough to induce detoxification gene expression but not too high that it acts as an insecticide. In order to determine the best caffeine exposure conditions, I tested a control genotype taken from the *smc5/6* study (*Iso*) at four caffeine concentrations: 0 mM, 2 mM, 8 mM and 16 mM to examine expression levels of *Cyp28a5, Cyp6a8, Cyp12d1 and CG16810* (Figure 3.2). This experiment serves a second purpose, because it validates that these detoxification genes are induced in wild type larvae, since the genes were selected from spotted cDNA microarray studies that were conducted in adults (*100*). To synchronize larval development, which ensures that gene responses are temporally aligned in a population, embryos were collected in one-hour intervals. Larval populations were reared on yeast paste plates for ~30 hours and then staged at the L2 to L3 instar molt. Healthy newly molted L3 larvae were placed on either fly media containing 2 mM, 8 mM, 16 mM caffeine or 0 mM controls for 4 hours before being collected in groups of 7 to 10 per sample. QPCR on mRNA extracted from these whole larvae showed significant increase in expression of all three CYP genes *cyp28a5, cyp6a8*, and *cyp12d1* as well as the GST gene *CG1681* upon caffeine exposure, but, as expected, this induction appeared to be dependent on the caffeine dosage.

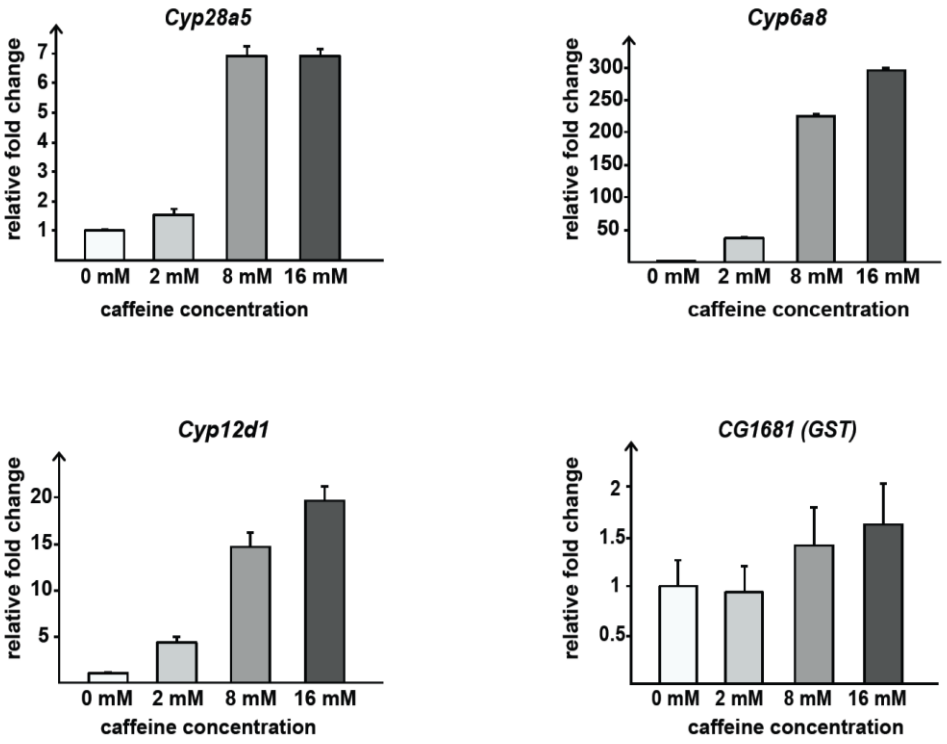


Figure 3.2 qPCR of known caffeine inducible genes showed that 2 mM, 8 mM and 16 mM caffeine were able to induce expression of detoxification genes as expected in 4 hr. old L3 larvae. *cyp28a5* was induced 1.6fold, 6.9fold and 6.9fold by 2 mM, 8 mM and 16 mM caffeine respectively. *cyp6a8* was induced 36.6fold, 224.7fold and 296.3fold by 2 mM, 8 mM and 16 mM caffeine respectively. *cyp12d1* was induced 4.4fold, 14.7fold and 19.7fold by 2 mM, 8 mM and 16 mM caffeine respectively. A GST gene *CG1681* was not induced by 2 mM caffeine (0.9fold) but rather slightly induced by 8 mM (1.4fold) and 16 mM (1.6fold) caffeine.

For example, *cyp6a8* was induced ~37fold by 2 mM caffeine, 225fold by 8 mM and the strongest induction was by 16 mM at an impressive ~300fold. The other two CYP genes behave similarly. The phase II detoxification GST gene *CG1681* was also induced by caffeine although it seemed less dosage dependent (Figure. 3.2). The differences in expression are more pronounced between 2 mM and 8 mM, less so between 8 and 16 mM. 16 mM was a very high caffeine concentration; larvae were unhealthy. Considering from all angles, I decided to use samples of young L3 larvae 4 hours on 8 mM caffeine in the subsequent microarray study. Taken together, this experiment determined that a concentration

of 8 mM caffeine triggered a robust, but non-toxic xenobiotic response in *Drosophila* larvae.

For the microarray, total RNA was extracted, reverse transcribed, biotin-labeled and hybridized to Affymetrix GeneChip Drosophila genome 2.0 microarray chips. A total of 6 chips were used as each condition tested in triplicate. We calculated expression values from the Affymetrix Raw files using the RMA software (*30*), and we used another software package, LIMMA (*95*), to determinefold changes and statistical significance. Both software packages were run on the statistical language "R". I focused my data analysis on the top caffeine-responsive genes (Fold change >2, $p<0.05$).

A total of 188 genes were upregulated, of which 16 were induced more than 10 fold. Members of all three classic detoxification gene groups are significantly overrepresented in the up-regulated gene set (Figure 3.3). These include twenty-five phase I detoxifying monooxygenases P450 enzymes ($p<6.6E-141$), thirteen GSTs ($p<5.3E-101$) and ten UGTs ($p<2.1E-60$) which are the phase II conjugative enzymes as well as two phase III ABC transporters ($p<0.00025$) (Table 3.2). The total number of induced genes with the Gene Ontology term oxidation-reduction reached 32 with a *p*-value of 1.14E-24. In addition, 13 general transport genes ($p<2.34E-05$) and 5 stress-related genes ($p<2.22E-08$) are overrepresented in the list of caffeine-upregulated genes (Table 3.2). A comparison with FlyBase transcriptome modENCODE RNA-Seq treatment data of the same 4 hours caffeine treatment (1.5mg/ml ≈ 8mM) (which was posted after I carried out my own microarray) to L3 larvae revealed subtle differences to

my Affymetrix microarray results (Table 3.3). The result showed similar correlations of the highly induced genes but not the lowly induced genes with some discrepancies. Of the P450 genes, *CG6870*, which had a fold change of 7.16, was listed as moderate; *Cyp6a20* (FC=3.66) was listed as low, as well as *Cyp6a22* (FC=2.13) as low. The top-induced *GstD5* and *Ugt86Dd* were listed as low despite having fold changes as high as 21.31 and 25.25 respectively. In fact, most UGTs were listed as low expression or 0 in the RNA-Seq data but were in the 2 to 25 fold changes in my microarray with high significance. Looking at the FlyAtlas organ/tissue specific expression data, the larvae tissue that is associated with the most genes induced by caffeine is the larval midgut. Out of the 79 induced detox genes (Table 3.3), 22 were found in the midgut. Fat body, Malpighian tubules and hindgut also had high numbers of detoxification gene expressed, the numbers being 19,14 and 11 respectively. This trend is true for phase I P450 genes as well as phase II GSTand UGT genes and phase III ABC transporters. Other tissues involved are trachea, salivary gland, CNS. These are less known for their function in insect detoxification but might have potential roles in detox response. Intriguingly, caffeine-treatment induced an entire family of genes characterized by a protein domain of unknown function (DUF): DUF227 (IPR004119). We found 22 DUF227 genes that were upregulated by caffeine, eight of which were induced more than 5 fold. There are only 41 DUF genes in the fly genome, demonstrating that the majority of DUF227 genes are transcriptionally induced by caffeine. One DUF227 gene *CG13360* was downregulated 2.5fold by caffeine (Table 3.4).

I found 154 genes that were downregulated upon caffeine treatment. Nine gene ontology terms or InterPro protein families were found highly enriched in the down-regulated gene set (Table 3.5). Twenty-three genes in total were found in the caffeine downregulated gene set with a known function in the regulation of protein translation. Caffeine down regulates ribosomal synthesis genes covering three GO terms: ribosomal RNA (rRNA) processing and modification genes ($p<6.42E-179$), genes involved in ribosomal assembly ($p<2.46E-16$) and Tif-IA ($p<4.05E-28$), a transcription regulator of RNA polymerase I, the only RNA polymerase that transcribes ribosomal RNA. As listed in Table 3.6, fourteen genes are associated with making mature ribosomal RNA. Six genes are essential for ribosome biogenesis. Tif-IA regulates the transcription of RNA pol I, which in turn regulates rRNA transcription. RNA-binding region RNP-1 (RNA recognition motif) and other nuclear splicing regulators (Table 3.7) were highly enriched as well ($p=2.11E-09$, $p=1.44E-12$ &1.26E-14 respectively). In addition to translation-related genes, transcription factors seemed to be negatively affected by caffeine ($p<0.0002$). Lastly the expression of transmembrane transport and chitin binding proteins were repressed ($p<1.79E-08$, $p<0.0007$).

Another way to look at representative gene ontologies that responded to caffeine in wild-type early third instar larvae is to focus on the top fifty differentially expressed genes (Table 3.8 and 3.9).

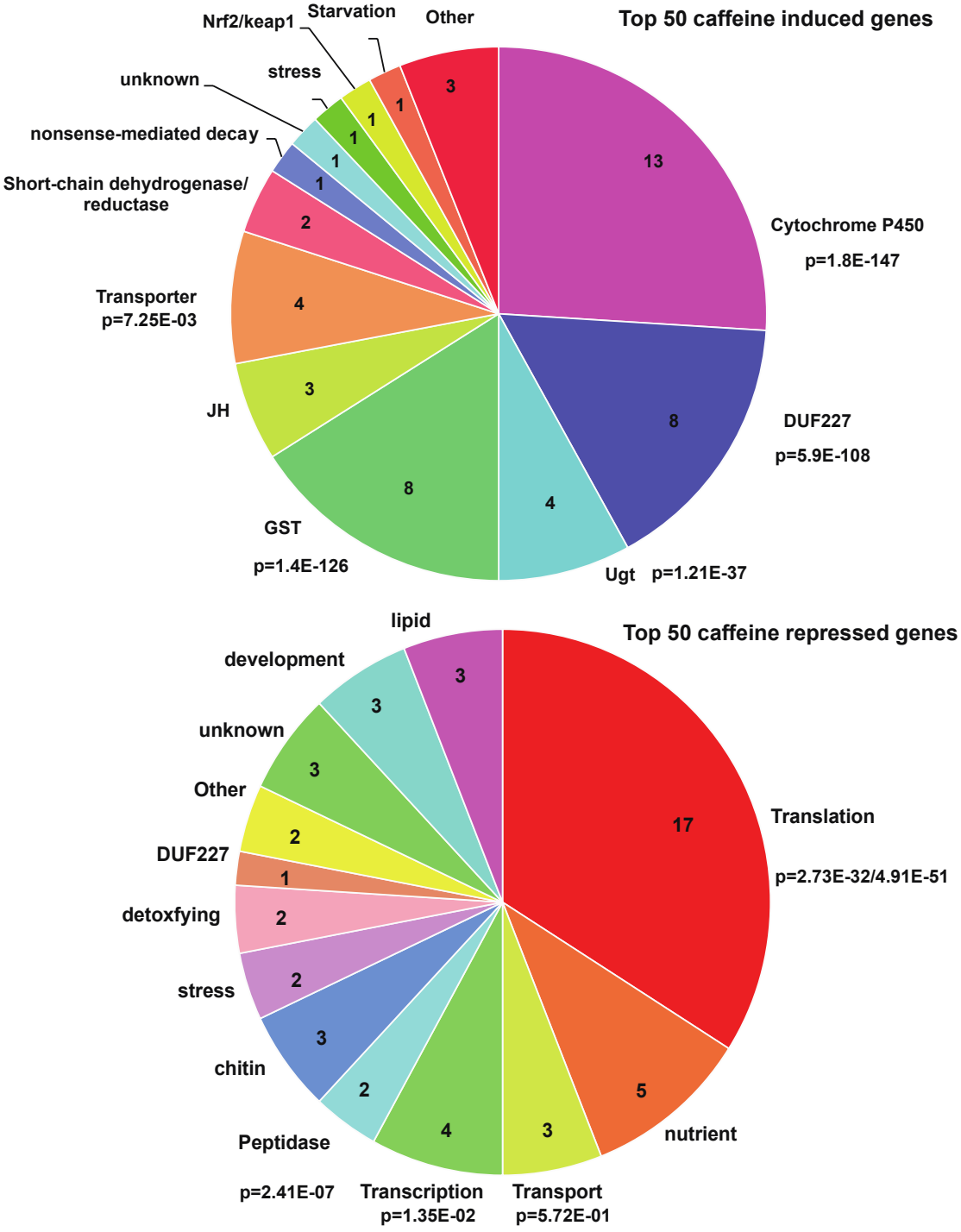


Figure 3.3 Pie charts of the top 50 differentially expressed transcripts upon caffeine exposure. The significance of enrichment was calculated by X^2 test. The significance of rRNA synthesis and ribosomal biogenesis was calculated separately and listed under translation category.

Amongst the top 50 genes induced by caffeine, 26 are genes encoding detoxification enzymes, 13 of which were P450 genes, 8 were GST genes, 4 UGT genes and one was an ABC transporter in the transporter category (Figure 3.3). Genes encoding detoxifying enzymes represented more than 50% of the top caffeine-induced gene set, clearly showing the effect of caffeine as a powerful xenobiotic response inducer. Other putative detoxification related genes include juvenile hormone epoxide hydrolases (3/50), transporters (4/50) and short-chain dehydrogenases/reductases (2/50). Given the high percentage of known detoxification genes in the list, it appears likely that most, if not all, of the other genes also have roles in detoxification processes. Finally, we have identified a component of the Nrf2/keap1 transcription cascade in our top 50 gene list, namely *keap1*, which is known to regulated oxidative and detoxification response in *Drosophila (96)*.

In the top 50 down-regulated genes, the largest group consists of seventeen translation-related genes (Figure 3.3), raising the question whether this is a general property of detoxification responses, or whether this is specific to the caffeine treatment. In the set of 154 significantly downregulated genes, the number of translation-related genes is twentythree (~15%). These include ribosomal RNA modification and processing genes, ribosomal protein methylation genes, transcripts for ribosome assembly and biogenesis as well as Tif-IA, the aforementioned factor that regulates transcription of the only RNA polymerase responsible for rRNA transcription, RNApol I (Table 3.8). The second largest group of down-regulated genes in the microarray consists of genes

associated with RNA splicing (Table 3.9). Interestingly, a small number of detoxification genes and genes encoding DUF227 domains are downregulated, although the majority of them are upregulated by caffeine showing that caffeine might differentially affect the same class of genes. Extracellular and intracellular transport is another GO term found in downregulated list. Even though general transcription factors are downregulated, this includes both positive and negative TFs thus how caffeine affect transcription through TFs is very quite complex, which reflected by our microarray results.

3.3.2 Caffeine microarray validation by high throughput 48.48 Fluidigm qPCR and testing 2 mM, 8 mM caffeine and starvation conditions with a subset of genes

One concern of using caffeine as a xenobiotic treatment is that caffeine is a bitter tasting substance, *Drosophila melanogaster* is known to avoid caffeine media when feeding, both adults and larvae (*89, 101*). The same avoidance behavior is true with other bitter tasting substances. It is considered an evolutionarily conserved mechanism of insects to avoid ingestion of toxins as they usually have a bitter taste. The most direct impact of caffeine avoidance on my experiment is that the caffeine treated larvae are slightly starved as many larvae were observed to leave the food and wander around or stay in the media but near the surface; this is an issue that cannot be remedied. In order to see whether starvation created by caffeine avoidance plays a role in mediating the transcriptional changes that we see in the microarray, I tested three genes from the downregulated gene set. A GCN5-related N-acetyltransferase *CG15155* is the top

downregulated gene, which is 12.4 fold down in the microarray. *Nop56*, which encodes a pre-mRNA processing ribonucleoprotein, is 3.6 fold downregulated. *mitochondrial ribosomal protein S10* that functions in hemolymph juvenile hormone binding is downregulated 3.3 fold in the microarray. The reason I only focused on the down regulated set of genes is that this was only a preliminary test to see if future experiments should include the starvation condition. The complete validation of microarray and testing the effect of starvation on gene expression is carried out in the 48.48 Fluidigm qPCR experiment. *CG15155* is down about 10 fold in both caffeine samples and starved samples (Figure. 3.4). *Nop56* is downregulated 4.2 fold by caffeine and down 2.27 fold by starvation. The difference of caffeine and starvation is more pronounced in *mRpS10*. Caffeine has no effect on *mRpS10* expression detected by qPCR thus the microarray result of *mRpS10* was not valid. *mRpS10* showed a 2.87 fold induction by starvation. In summary, there was evidence that caffeine-mediated differential gene expression was impacted by starvation in some cases to the same degree and in other cases varies significantly. This warrants starvation as a plausible condition to be tested along side caffeine treatment in the future experiments.

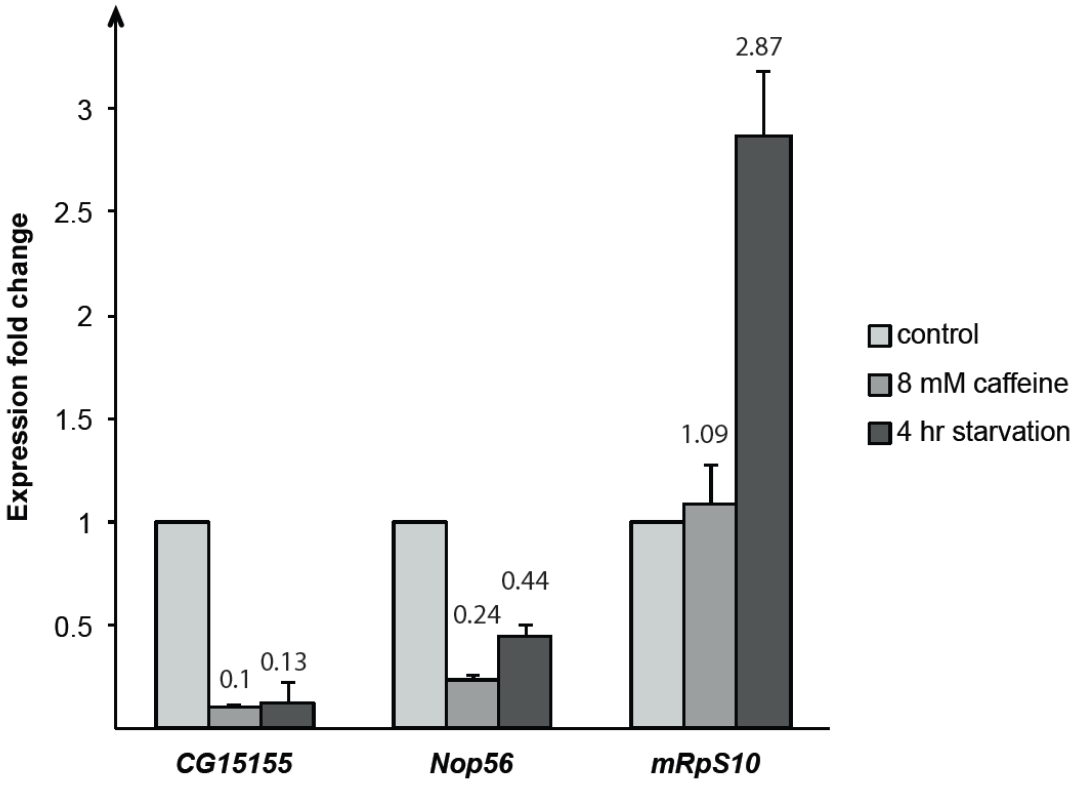


Figure 3.4 Comparison of gene expression changes brought about by 4 hour 8 mM caffeine treatment or 4 hour amino acid starvation by quantitative real-time PCR in a subset of genes: *CG15155*, *Nop56* and *mRpS10*.

In order to validate the caffeine microarray result with a large-scale qPCR approach, I used a high throughput gene expression platform (Fluidigm BioMark™) to confirm fold changes of differentially expressed genes. The idea was to create a representative gene set for xenobiotic responses, which could be used to test a range of conditions that were not tested in the original microarray. Such conditions could include starvation, and in particular, RNAi lines or mutants, to test whether any of the genes would be dependent on such changes. The BioMark™ is based on proprietary microfluidic chips called integrated fluidic circuits (IFCs) that allow simultaneous detection of up to 9216 real time

qPCR reactions on a single chip with the conventional Taqman® or Roche probe chemistry (*102, 103*). It is a very powerful and convenient technology for mid- to high throughput targeted gene expression studies. I used the 48 by 48 array and 96 by 96 array formats for my high throughput qPCR experiments. To cover all bases, I selected candidate genes based on fold changes that span the entire expression spectrum of each enriched gene ontology class, meaning that for each enriched ontology class, I picked candidates with high/medium/low expressions where applicable to cover the whole range in the microarray with a special emphasis on genes with high fold changes (Table 3.10).

Gene ontology classes tested in the up-regulated gene set are genes encoding cytochrome P450s, GSTs, ABC transporters, DUF227 proteins, metabolism genes and other specific genes based on their protein domains (Figure 3.5A). Two of the three P450 genes were induced specifically by caffeine in a dose-dependent manner. The top up-regulated gene in the microarray is *Cyp6a8*, which was 61 fold upregulated ($p<5.73E-11$). The qPCR experiment showed that caffeine induced the expression of *Cyp6a8* 20 fold at 2 mM concentration and about 100 fold at the 8 mM concentration. On the other hand, 4-hour starvation results in a 3.6 fold decrease in *Cyp6a8* expression when compared with controls. *Cyp6a21* appears to be similar to *Cyp6a8*: starvation seemed to induce the highest *Cyp6a2* expression, followed by 8 mM caffeine. This finding is quite unusual as *Cyp6a2* is a well-established detoxification P450 gene. The GST, ABC transporter and DUF227 genes tested all exhibited highestfold-induction by 8 mM caffeine with the exception of ABC transporter *l(2)03659*. The difference of

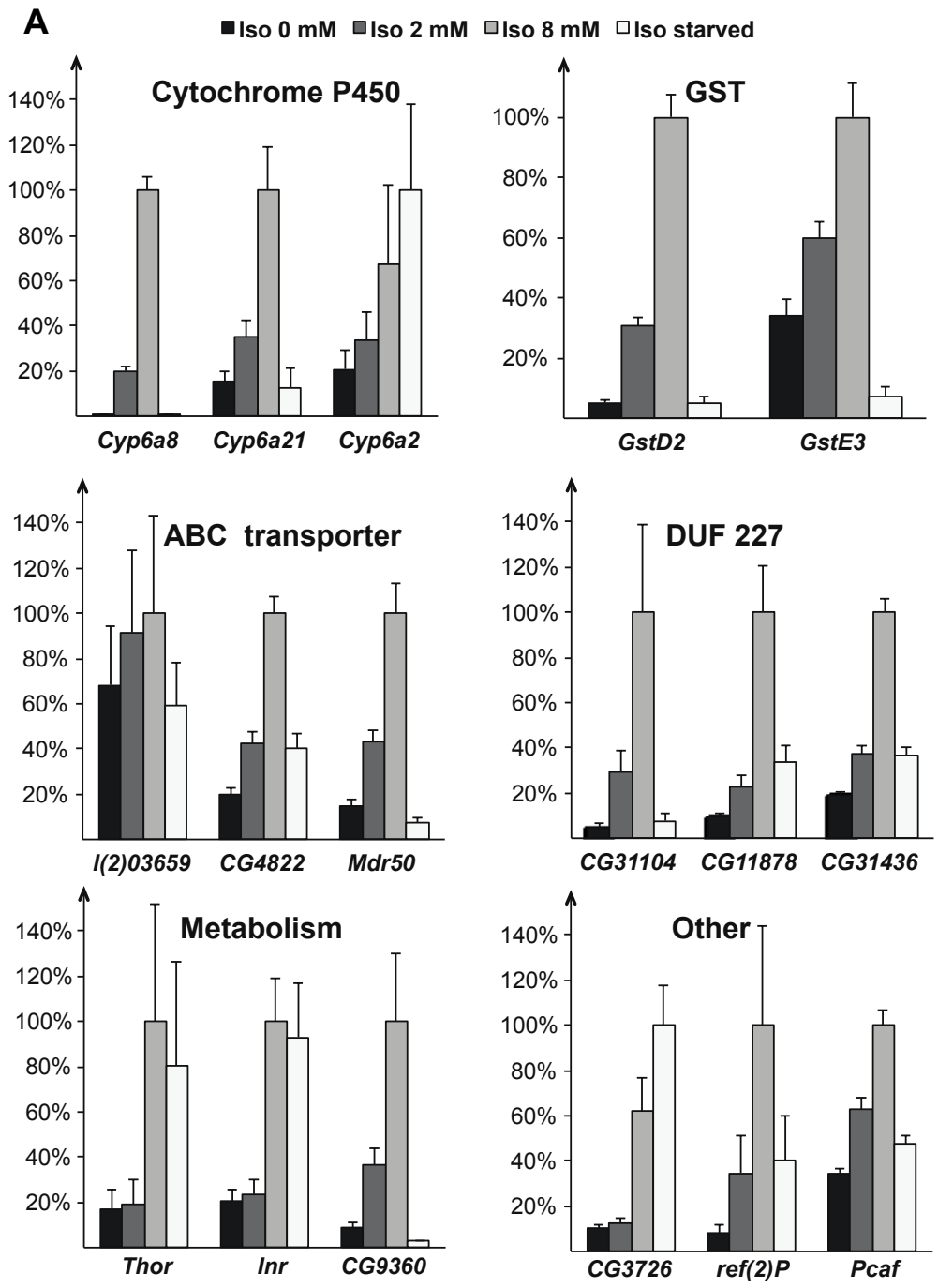
l(2)03659 expression levels in the four conditions is not pronounced, with overlapping error bars possibly indicating a pair of failed primers. Amongst metabolic genes, *Thor*, which encodes the *Drosophila* Insulin stimulated eIF-4E binding protein and *Insulin receptor* (*Inr*) were induced by 8 mM caffeine as well as starvation to a similar degree. *CG9360* is a gene that encodes a short chain dehydrogenase/reductases that may have a function in metabolizing xenobiotics. It is induced only by caffeine in a dosage dependent manner. In fact, all the GO classes that deal with detoxification are induced by caffeine but not starvation, except *Cyp6a2*, showing that the transcriptome changes depicted by the microarray was mostly reflecting a caffeine-induced detoxification response. Other upregulated genes validated by the 48.48 Fluidigm qPCR are *CG3726*, *ref(2)P* and *Pcaf*. *CG3726* has a BTB/POZ domain that is an evolutionarily conserved protein-protein interaction domain for dimerization or oligomerization; it is an extended sequence motif to C_2H_2 zinc finger or the Kelch motif found in some actin-binding proteins. The BTB/POZ domain is seen in the co-repressors of nuclear receptors such as PXR or CAR in one model (*104*). The nuclear pregnane X receptor (PXR) and constitutive androstane receptor (CAR) in the murine model are known to regulate genes involved in all three phases of xenobiotic detoxification (*105*). *CG3726* is induced 6 fold by 8 mM caffeine and 10 fold by starvation. The gene *ref(2)P* has an octicosapeptide/Phox/Bem1p domain or OPCA motif containing PB1 domain for short. PB1 domains serve as dimerization/oligomerization domains in adaptor proteins or kinases that are very important players in cellular signaling pathways. Examples of PB1 domain

containing kinases are aPKC, p62, MEKK2/MEKK3, MEK5 and Par-6; they play crucial roles in critical cellular processes such as osteoclastogenesis, angiogenesis, early cardiovascular development or cell polarity (*106*). *Pcaf* is a Gcn5 related N-acetyltransferase. GCN5 is a ubiquitous histone acetyltransferase that activates gene transcription by adding acetyl groups to core histone. This gene is renamed as *Gcn5 ortholog* on FlyBase and it is referred to as *Gcn5* hereafter. Both *ref(2)P* and *Gcn5* are induced by 8 mM caffeine 12.5 and 2.9 fold but not so much by starvation.

For the validation of downregulated genes, I tested ribosomal biogenesis genes, mRNA splicing genes, genes encoding brix domains or WD-40 repeats, as well as transcription factor *Krüppel* and *diminutive* (i.e. *Myc*). The highly repressed genes *CG11425* and *ninaD* were also tested (Figure 3.5B). The top repressed gene *CG15155* was tested in the Fluidigm but the qPCR reaction failed due to noncompliant primers so it was not included in the graphs. Of all the genes tested, ribosome biogenesis gene *NHP2* and transcription factor *Myc* did not show caffeine-dependent or starvation-induced fold-reduction. Pre-mRNA-splicing genes and the other three ribosomal biogenesis genes are repressed by caffeine and starvation to a similar effect. Brix domain family proteins are a key to the ribosomal biogenesis pathway and rRNA binding (*107*). The brix domain *CG11583* and *peter pan* showed an equivalent fold-reduction by 8 mM caffeine and starvation but interestingly not *Myc,* which promotes rRNA synthesis by directly regulating RNA pol I transcription (*108*). WD40 repeats represent a motif important for multi-protein complex assembly or serve as a scaffold for protein

interaction. WD40 is enriched in the downregulated set of my caffeine microarray. WD40 containing *CG3071*, *CG7845* and *CG30349* are repressed by both 8 mM caffeine and starvation. The same goes for *ninaD*, but it was about -14 fold by 8 mM caffeine and -16 fold by starvation. The transcription factor *Krüppel* is one of the two genes that were downregulated by caffeine specifically. *Krüppel* is a gap gene that represses transcription of other genes. Caffeine downregulated *Krüppel* 4.8 fold but starvation only 2.2 fold. The other caffeine specifically repressed gene is a PA-phosphatase related phosphoesterase *CG11425* that was downregulated -4.3 fold in the microarray. Caffeine reduced *CG11425* to -5.5 fold however starvation repressed only 1.4 fold.

In summary, the microarray results of caffeine dependent differential fold changes almost perfectly correlated with the qPCR result, with the exception of induced ABC transporter *l(2)03659* and repressed ribosome biogenesis gene *NHP2*. The microarray underestimated the fold changes compared with the qPCR. Starvation did not contribute to the massive induction of detoxification genes or DUF227 genes. It is however the reason for the induction of *Thor* and *Inr* and repression of ribosome biogenesis and related genes, also mRNA splicing and WD-40 genes. In other words, there is likely a caffeine-induced xenobiotic detoxification transcriptional regulator or regulator network that is independent of starvation response. Starvation and caffeine both contributed to the down regulation of ribosomal biogenesis and other translation associated genes as well as pre-mRNA splicing genes.


A
Iso 0 mM
Iso 2 mM
Iso 8 mM
Iso starved
Cytochrome P450
140%
120%
100%
80%
60%
40%
20%
Cyp6a8
Cyp6a21
Cyp6a2
GST
100%
80%
60%
40%
20%
GstD2
GstE3
ABC transporter
140%
120%
100%
80%
60%
40%
20%
l(2)03659
CG4822
Mdr50
DUF 227
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80%
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40%
20%
CG31104
CG11878
CG31436
Metabolism
140%
120%
100%
80%
60%
40%
20%
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CG3726
ref(2)P
Pcaf

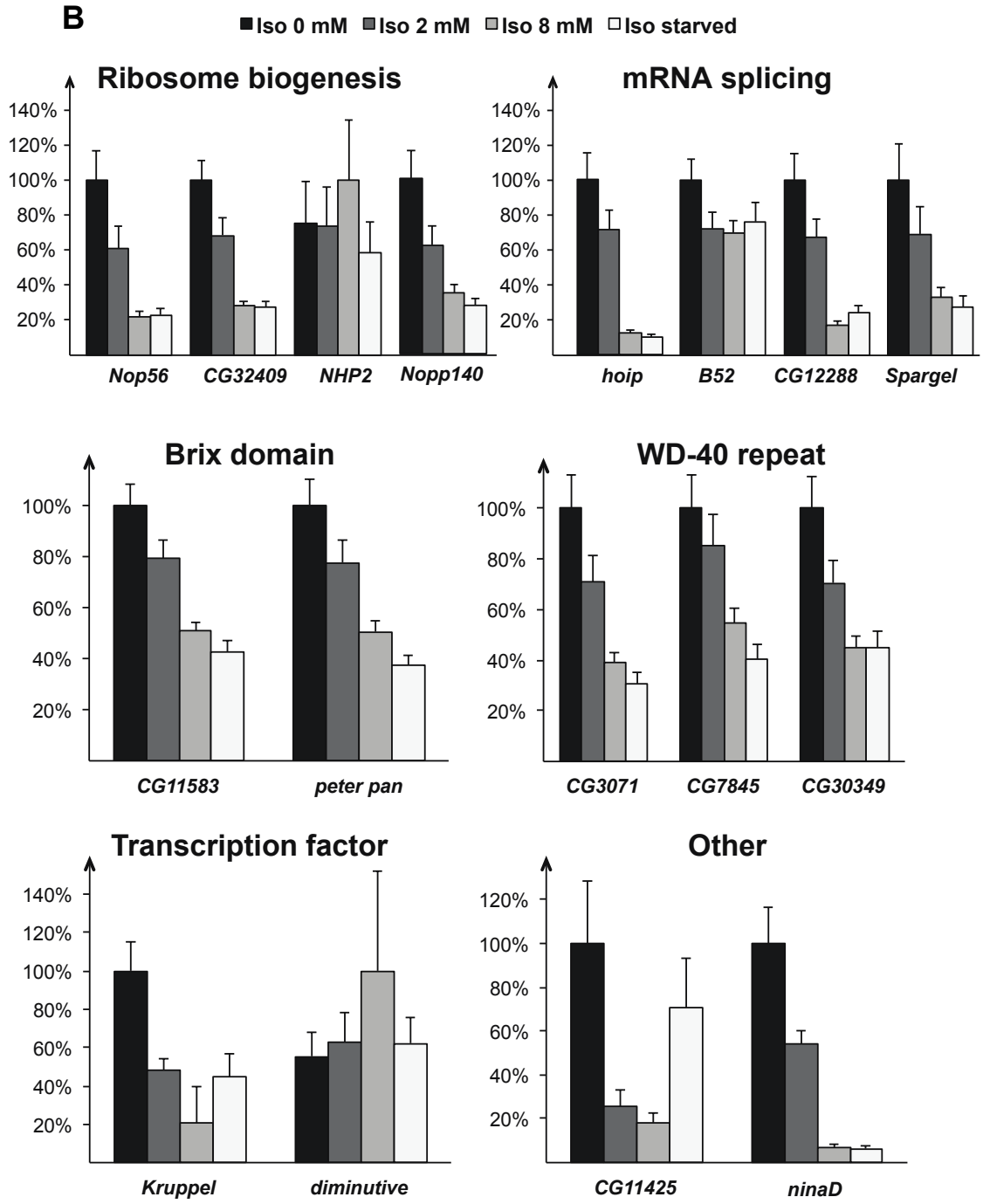


Figure 3.5 Validation of differentially expressed genes in caffeine microarray by 48.48 Fluidigm qPCR. Each gene was tested in 0 mM, 2 mM, 8 mM caffeine treated 4 hours old L3 and 4 hours L3 in starvation conditions. The fold changes for each gene were normalized as a percentage of the max expression of the particular gene.

3.3.3 Comparison of caffeine microarray with Phenobarbital, *DHR96-cDNA*, *dNrf2* over-expression and starvation microarrays

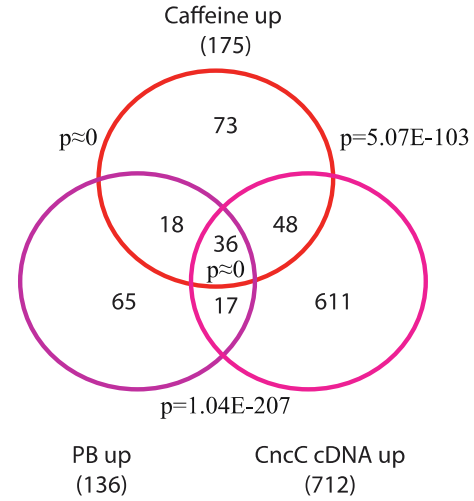
To find a common set of detoxification genes, data from the caffeine microarray was compared to a previously published phenobarbital (PB) microarrays results(reviewed by*109*). There was significant overlap (53 genes, p< 4E-281) with the phenobarbital-induced genes (208), showing induction of a common set of detoxification genes by the two xenobiotics likely under the control of common transcription regulators (data not shown). It is plausible to expect that considering caffeine and PB are both bitter tasting chemicals that caused the larvae to avoid the drug-containing media; reduced food intake could trigger starvation-mediated transcription response. On the other hand, *DHR96* and the *Drosophila Nrf2 (NF-E2-related factor 2)* ortholog *(cap 'n' collar isoform-C) CncC* (hereafter referred to as *dNrf2*) are transcription factors associated with transcriptional regulation of xenobiotics detoxification responses (*56, 89*). Manipulation of levels of *DHR96* or *Nrf2* leads to predicted alterations of xenobiotic-inducible gene expressions in the flies (*56, 89*). I would therefore argue that there is a common set(s) of genes differentially regulated by multiple pathways/drug treatments. To assess the commonality of transcriptional responses amongst treatment PB, caffeine and starvation in wild type or genotype *DHR96-cDNA/ dNrf2-cDNA* and get a clear picture of what is caffeine specific, cross comparisons of the respective microarray data were carried with Microsoft Access®.

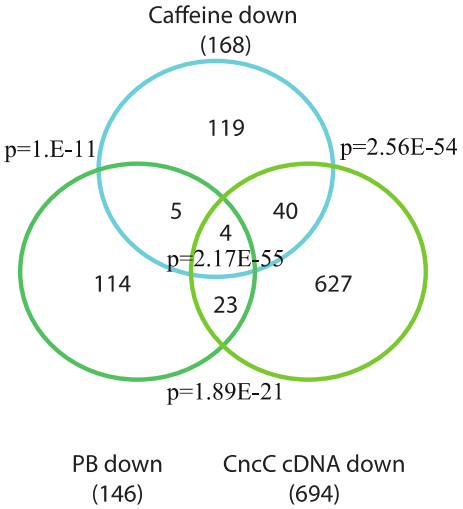
Firstly, the caffeine-array was compared with the PB and ectopic *Nrf2* (*CncC-cDNA*) microarrays. This was to get a common differentially expressed gene set that are most likely to represent a true set of xenobiotic-inducible detoxification genes. As expected, a large number of genes are present in the common upregulated set (n=36, p≈0). These include 9 P450 genes, 7 GST and 5 UGT genes, 1 ABC transporter, 3 short-chain dehydrogenase/reductases, 2 Jheh that respond to toxin, 6 DUF227 domain encoding genes, 2 acyl-CoA dehydrogenases and Keap1. Each and every class mentioned above is associated with insect detoxification. Indeed a core xenobiotic detoxification response was elicited by caffeine or PB treatments that was also induced by overexpressing *Nrf2*. However, DHR96 seemed to induce a different set of detox genes from *Nrf2* (Figure 3.6B). Caffeine, PB, *DHR96* and *Nrf2* induced two common detox genes *Cyp6a2* and *CG2065* (comparing Figure 3.6A and B). Ectopic expression of *DHR96* represses genes associated with xenobiotic and metabolic pathways (*56, 89*). *CG15155*, a N-acetyl transferase is the only common gene downregulated by caffeine, PB, *DHR96* and *dNrf2*. *CG15155* is also the top repressed gene in my caffeine microarray. Given that N-acetyl transferase is a histone modifier that potentially influences gene expression through chromatin modification, *CG15155* is an interesting candidate to follow up.

Secondly, in order to get a clear picture of what common detox associated genes were affected by starvation-mediated responses, cross comparisons were done with caffeine, PB and starvation (Figure 3.6C). The starvation data set is derived from differentially expressed genes that were common to two independent

starvation studies that looked at dietary amino acid starvation or starvation as a reference to sugar dependent gene regulation (*110, 111*). Five genes were in the up-regulated overlap; four of which are P450 genes, including the prominent *Cyp6g1*, which is strongly associated with DDT resistance (*56*). This suggests an unobserved phenomenon where starvation created stress is linked to detoxification pathways. This is further supported by the fact that 4 detox genes and 2 stress-associated genes were amongst the 9 common genes in the induction-overlap of caffeine, ectopic *Nrf2* and starvation (Figure 3.6D). *CG6330* is a gene that was over-transcribed in all four conditions. It encodes a uridine phosphorylase. Down regulation of ribosome biosynthesis genes is a common theme for caffeine, ectopic *Nrf2* and starvation but not PB. Caffeine inhibits TOR kinase, which shuts down protein synthesis in response to starvation (*112*), suggesting that the down regulation of ribosomal biosynthesis genes could be the result of TOR inhibition.

A





CncC overexpression, caffeine or PB treatment overlap up regulated genes

Gene	FC	p-value	Function
Cyp6a8	60.71	5.73E-11	cytochrome P450
Cyp6w1	30.91	8.07E-08	cytochrome P450
Ugt86Dd	25.25	1.42E-07	UDP-glucuronosyltransferase
CG6908	21.62	4.49E-07	Protein of unknown function DUF227
CG2065	17.99	3.95E-07	Short-chain dehydrogenase/reductase SDR
Cyp6a2	16.79	8.20E-08	cytochrome P450
CG5724	15.35	1.18E-07	UDP-glucuronosyltransferase
GstD2	12.02	1.24E-09	glutathione transferase
Cyp4d14	10.41	9.09E-07	cytochrome P450
Cyp6d5	9.26	1.40E-07	cytochrome P450
Mdr50	8.38	8.77E-09	ABC transporter
GstD7	7.80	4.07E-06	glutathione transferase
CG31288	7.26	7.57E-08	Protein of unknown function DUF227
Jheh2	6.73	4.98E-08	epoxide hydrolase response to toxin
CG9360	6.66	1.49E-05	Short-chain dehydrogenase/reductase SDR
Keap1	6.06	6.06E-07	BTB/POZ domain, Kelch repeats, inhibitor of dNrf2
Cyp4p1	5.67	9.34E-06	cytochrome P450
Cyp6a21	5.58	7.56E-09	cytochrome P450
Jheh1	4.95	4.26E-08	epoxide hydrolase response to toxin
GstE1	4.78	1.16E-07	glutathione transferase
GstE3	4.13	4.53E-05	glutathione transferase
Acox57D-p	4.12	2.50E-06	acyl-CoA dehydrogenase
CG2064	3.85	2.13E-07	Short-chain dehydrogenase/reductase SDR
CG10553	3.77	1.59E-06	Protein of unknown function DUF227
CG10560	3.70	3.19E-07	Protein of unknown function DUF227
Cyp4e2	3.54	4.06E-07	cytochrome P450
Cyp6g1	3.02	8.71E-03	cytochrome P450
GstE7	2.97	4.00E-03	glutathione transferase
GstE5	2.74	4.85E-03	glutathione transferase
Ugt86Dc	2.69	1.59E-06	UDP-glucuronosyltransferase
CG13658	2.66	1.76E-05	Protein of unknown function DUF227
CG5009	2.51	1.63E-05	acyl-CoA dehydrogenase
CG9498	2.37	2.44E-05	Protein of unknown function DUF227
Ugt37b1	2.25	1.30E-03	UDP-glucuronosyltransferase
GstE6	2.17	9.46E-06	glutathione transferase
Ugt36Ba	2.14	7.51E-04	UDP-glucuronosyltransferase

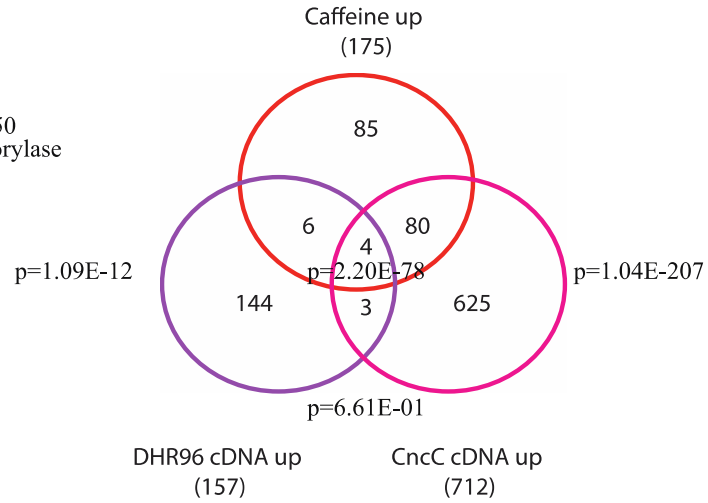
CncC overexpression, caffeine or PB treatment overlap down regulated genes

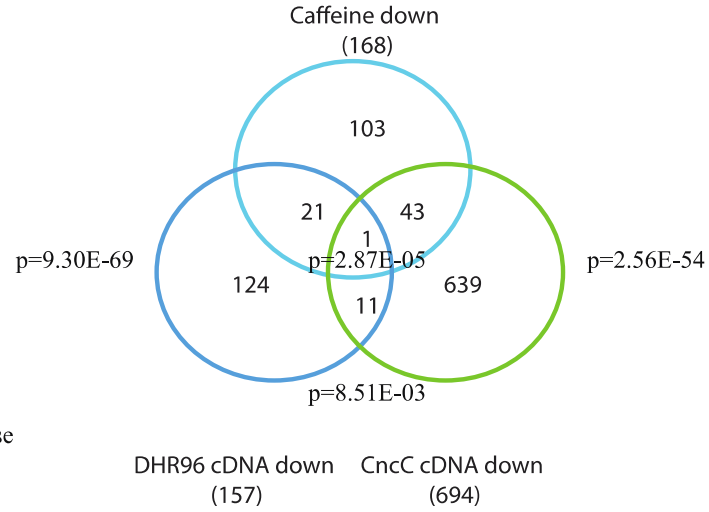
Gene	FC	p-value	Function
CG15155	-12.40	5.69E-06	N-acetyltransferase
CG8745	-3.88	1.05E-05	response to nicotine
Lsd-1	-2.53	1.95E-03	lipid storage
CG8791	-2.28	5.78E-03	transmembrane transport

B

caffeine, CncC or DHR96 overexpression overlap up regulated genes

Gene	FC	p-value	Function
CG2065	17.99	3.95E-07	oxidoreductase
Cyp6a2	16.79	8.20E-08	cytochrome P450
CG6330	2.50	9.25E-04	uridine phosphorylase
CG34165	2.43	6.58E-03	unknown





caffeine, CncC or DHR96 overexpression overlap down regulated genes

Gene	FC	p-value	Function
CG15155	-12.40	5.69E-06	N-acetyltransferase

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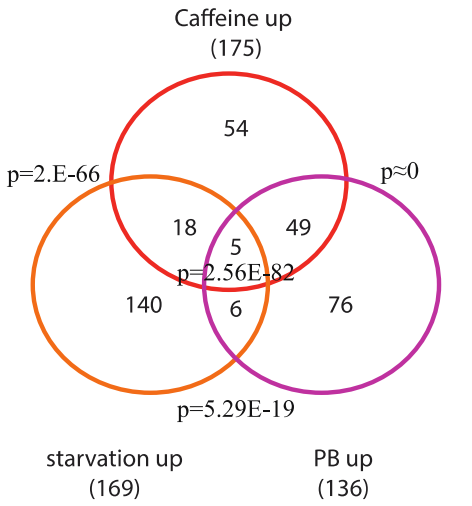
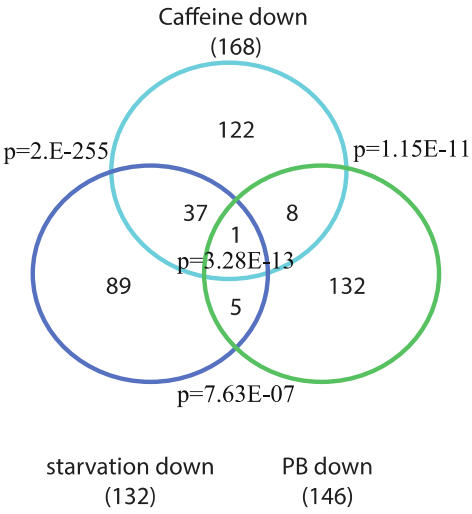


starvation, caffeine or PB treatment overlap up regulated genes

Gene	FC	p-value	Function
Cyp6d5	9.26	1.40E-07	cytochrome P450
Cyp12a5	5.55	2.05E-04	cytochrome P450
CG15784	3.17	1.11E-03	unknown
Cyp6g1	3.02	8.71E-03	cytochrome P450
Cyp6a14	2.99	8.63E-05	cytochrome P450



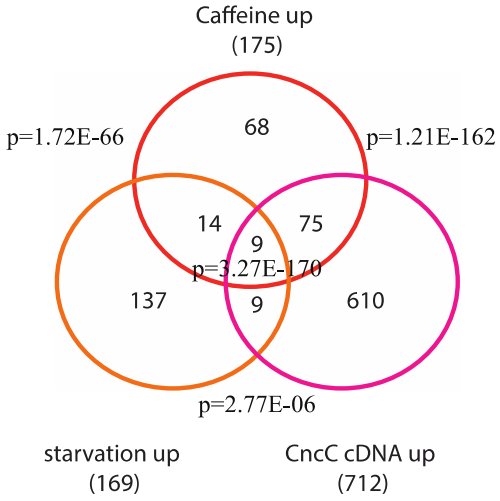
starvation, caffeine or PB treatment overlap down regulated genes

Gene	FC	p-value	Function
obst-A	-2.85	1.81E-05	Chitin binding Peritrophin-A

D

caffeine, starvation or CncC overexpression overlap up regulated genes

Gene	FC	p-value	Function
CG5999	10.33	1.96E-06	glucuronosyltransferase
Cyp6d5	9.26	1.40E-07	cytochrome P450
Smg5	7.02	6.77E-06	nonsense-mediated decay
CG1882	5.28	7.04E-08	carboxylesterase
CG9009	3.23	2.74E-03	long-chain-fatty-acid-CoA ligase
Cyp6g1	3.02	8.71E-03	cytochrome P450
l(2)efl	2.94	1.94E-03	response to stress
CG6330	2.50	9.25E-04	uridine phosphorylase
PHGPx	2.07	6.21E-06	response to oxidative stress



caffeine, starvation or CncC overexpression overlap down regulated genes

Gene	FC	p-value	Function
CG12288	-3.72	8.93E-04	RNA-binding region RNP-1
Nop56	-3.60	7.62E-05	Pre-mRNA processing ribonucleoprotein
Nopp140	-3.58	5.73E-05	Treacle-like, Treacher Collins Syndrome
CG7637	-2.78	5.88E-04	rRNA processing
CG8545	-2.65	7.94E-04	rRNA processing
nop5	-2.36	1.25E-03	rRNA processing
CG9253	-2.31	3.35E-04	metal ion transport
CG13900	-2.25	1.17E-03	nuclear mRNA splicing
CG9246	-2.17	6.68E-05	rRNA processing
r-l	-2.08	1.81E-03	UMP synthase
CG8939	-2.05	1.44E-03	rRNA processing

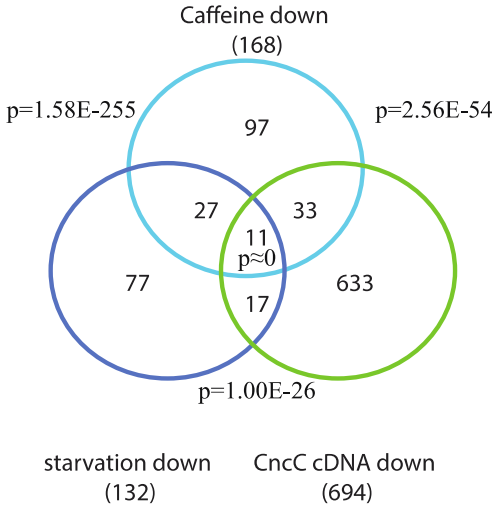


Figure. 3.6 Cross comparison of caffeine, PB, CncC-cDNA, DHR96-cDNA and starvation microarray data. Venn diagrams illustrate overlaps of the three data sets at a time, showing up- and downregulated genes. All overlaps are highly statistically significant. The common genes affected by all three conditions are listed according tofold changes. (A) comparison amongst caffeine, PB and CncC-cDNA for common induced detox gene set and common detox repressed gene set. (B) comparison amongst caffeine, PB and DHR96-cDNA for common induced and repressed detox gene set. (C) comparison amongst caffeine, PB and starvation for common starvation activated detox genes and starvation repressed ribosomal biosynthesis genes. (D) comparison amongst caffeine, CncC-cDNA and starvation for starvation activated detox genes and starvation repressed common gene set.

3.3.4 Analysis of caffeine-sensitive mutants as well as mutants in the cAMP/PKA and JNK pathway for defects in the detoxification response

Defining a true set of xenobiotic induced genes would help us characterize the xenobiotic response in arthropods. Cross comparisons of caffeine microarray and other detox or starvation microarrays has shown that P450s, GSTs, reductases, ABC transporters, DUF227 and juvenile hormone metabolizing enzymes are truly transcriptionally induced detoxification genes, whereas ribosome biogenesis and ecdysone biosynthesis genes were repressed by caffeine administration and starvation. In fact, the majority of the xenobiotic-responsive detoxifying enzymes are induced in a starvation independent manner (Fig 3.5A and 3.7 D-K). In turn, testing for failure in properly inducing this gene set in certain caffeine sensitive mutants would show us defects in eliciting a xenobiotic response because of the genetic lesion in the molecular pathways. Two to five representative genes of each class mentioned above were chosen, their expressions quantified with Fludigm 96.96 real-time qPCR. More importantly, differentially expressed transcription regulators and top affected genes were tested to look for clues that point to the transcription factor/network that regulates detoxification response (Table 3.11). Caffeine is considered a natural pesticide albeit no caffeine-based insecticide products are currently found on the market. As shown by my caffeine microarray, caffeine elicits a genome-wide transcriptional response at 8 mM concentration. Similar concentrations 1.5 mg/ml (=7.724 mM) close to concentration that I used have been also utilized in other detoxification studies (*30, 98*). Higher caffeine concentration of 16 mM that was

used before in luciferase induction experiments was not selected due to the fear of elevated level of starvation (*99*). Such a concentration does not cause complete lethality in wild type *D. melanogaster*. Intriguingly, three caffeine sensitive mutants *jnj (java-no-jive)*, *sst (sleepless-in-seattle)* and *ddt (double-double-trouble)* were isolated in an EMS screen in search of DNA damage repair mutants (see chapter 2). These mutants are extremely sensitive to the effects of caffeine at a concentration as low as 2 mM. The pre-mutagenized isogenized wild type stock for this screen was designated *Iso*. In order to understand the underlying mechanism of this apparent caffeine sensitivity, we tested whether mutant mortality was associated with unsuccessful detoxification of caffeine by measuring the changes in detox gene expressions (Table 3.11). Because 2 mM caffeine was used to select mutants in the caffeine screen, both 8 mM and 2 mM were included in the 96.96 Fluidigm qPCR along with starvation.

Gene expression was first quantified in *Iso* 4 hr L3 samples collected on standard media, 2 mM caffeine, 8 mM caffeine and 4-hour starvation (Fig 3.7). Top caffeine repressed genes *ninaD*, *CG11425* and *Nopp140* and transcription factor *Krüppel* were tested because the drastic expression changes can give me insights to caffeine's function. PA-phosphatase related phosphoesterase *CG11425* was downregulated in a caffeines-specific manner, meaning that starvation had no effect on its expression. On the other hand, scavenger receptor *ninaD,* transcription factor *Krüppel* and *Nopp140,* which plays an important role in neurogenesis and its ortholog in human is associated with Treacher Collins Syndrome, responded to starvation in addition to caffeine (Figure 3.7B).

Translation-related genes *CG3071, CG32409, CG12288* and *spargel* are downregulated by caffeine in a dose- dependent manner; starvation has a similar effect on these genes as 8 mM caffeine. Cytochrome P450 enzymes *spok* and *phm*, which both mediate ecdysone biosynthesis, seem to respond to caffeine and starvation in opposite fashion: Caffeine did not alter the expression level of spok, whose expression increased in starvation. On the other hand, *phm* is repressed by caffeine but induced by starvation (Fig 3.7C). The commonality here is starvation induced *spok* and *phm* expression. Other P450s that presumably are detoxification-specific are highly induced by caffeine (Figure 3.7D). *Cyp6a8, Cyp6w1, Cyp6d5* and *Cyp6a21* are solely caffeine-responsive, but do not respond to starvation. *Cyp6a20* and *Cyp6d4* are induced mainly by caffeine; the effect of starvation is limited. In the case of *shd* and *Cyp6g1*, starvation-induced their expression as high as 8 mM caffeine. Surprisingly, the highest induction of *Cyp6a2* came from starvation. I asked the question: is this a result of stress created by starvation alone? Could *Cyp6a2* represent a new class of P450s that responds to starvation stress but not a particular xenobiotic? This phenomenon was explored further in section 3.3.4. Consistent with the idea that caffeine is able to induce a wide range of key detoxification enzymes that were not the result of simple starvation, GST genes (*GstD2*, *GstE3*, *GstE7*), ABC transporters (*CG4822*, *Mdr50*), reductases (*CG9360, CG2065, CG1441*), DUF227 genes (*CG31104, CG11878, CG31436*) and genes respond to juvenile hormone (*Jheh1, Jheh2, Jhl-26*) were highly induced by caffeine independent of starvation (Figure 3.7E-H and K).

In search for transcription factors that could potentially regulate the up regulation of the key detoxification enzymes, I tested for induction of putative DNA-binding proteins *CG3726*, which has a BTB/POZ domain; *Pcaf*, which is the fly homolog of the yeast GCN5-related N-acetyltransferase (hereafter referred to as *Gcn5*); and *CG10916*, which has a zinc-finger and a RING domain (Figure 3.7I). *CG3726* is largely starvation-induced whereas *Gcn5* and *CG10916* are induced by caffeine. Currently, there are two transcription regulators *DHR96* and *dNrf2* that have established roles in xenobiotic responses. The BTB/POZ domain containing inhibitor of *dNrf2*, *Keap1*, which received its name from its signature Kelch repeats, was also highly induced by caffeine. Other highly induced candidates from the caffeine microarray includes *ref(2)P,* which has a function associated with viral infectious cycle, *Smg5,* which mediates nonsense mediated decay, *l(2)efl,* which is heat shock protein Hsp20 as well as *Dgp-1* which encodes elongation factor Tu.

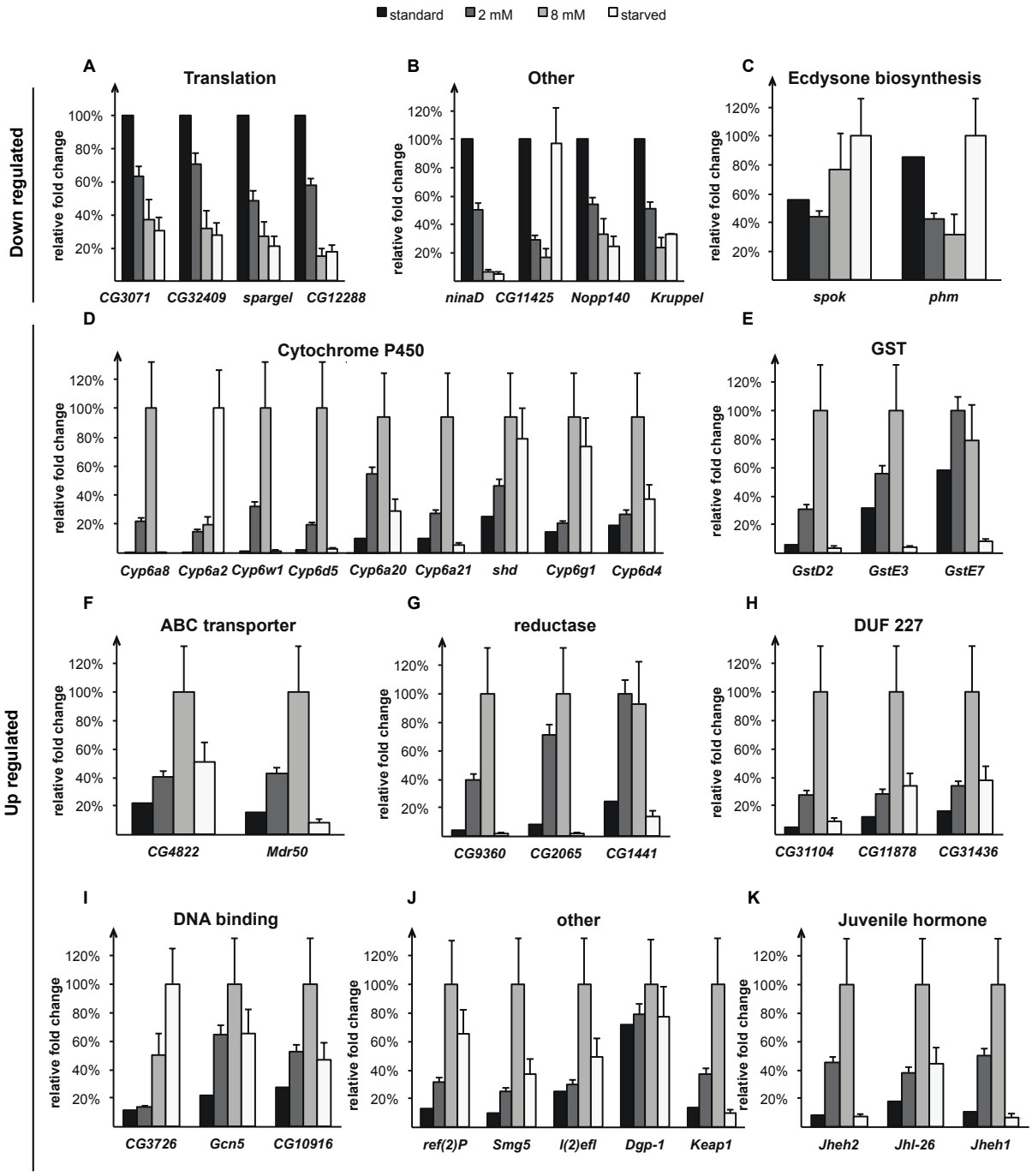


Figure 3.7 Fluidigm qPCR testing of presumptive caffeine-responsive detoxification genes in the presence of 2 mM and 8 mM caffeine, as well as starvation. (A-C) downregulated genes (D-K) upregulated genes.

Caffeine represents chemical stress to the organism, highly induces various P450 genes in *Drosophila (25).* The molecular basis of this induction is unclear, except for the fact that caffeine induction of *Cyp6a8* and *Cyp6a2* is mediated through cAMP pathway and involves the D-jun protein. The upstream regions of *Cyp6a8* and *Cyp6a2* contain many cis-elements that are predicted binding sites for "cAMP response element binding protein" (CREB), CRE-BP and AP1, the very transcription factors associated with cAMP and D-jun mediated transcription regulation (*30, 99*). D-jun is the *Drosophila* Jun-related antigen; it dimerizes with its binding partner Fos-related antigen or D-fos to form AP-1 early response transcription factor. AP-1 is activated through double phosphorylation by the JNK (*113*). JNK pathway is activated by stress stimuli such as UV irradiation, heat shock (*114)* or osmotic shock. Caffeine could be another stress that induces JNK signaling to turn on P450 genes. The *Drosophila* homolog of JNK is *basket* (bsk).

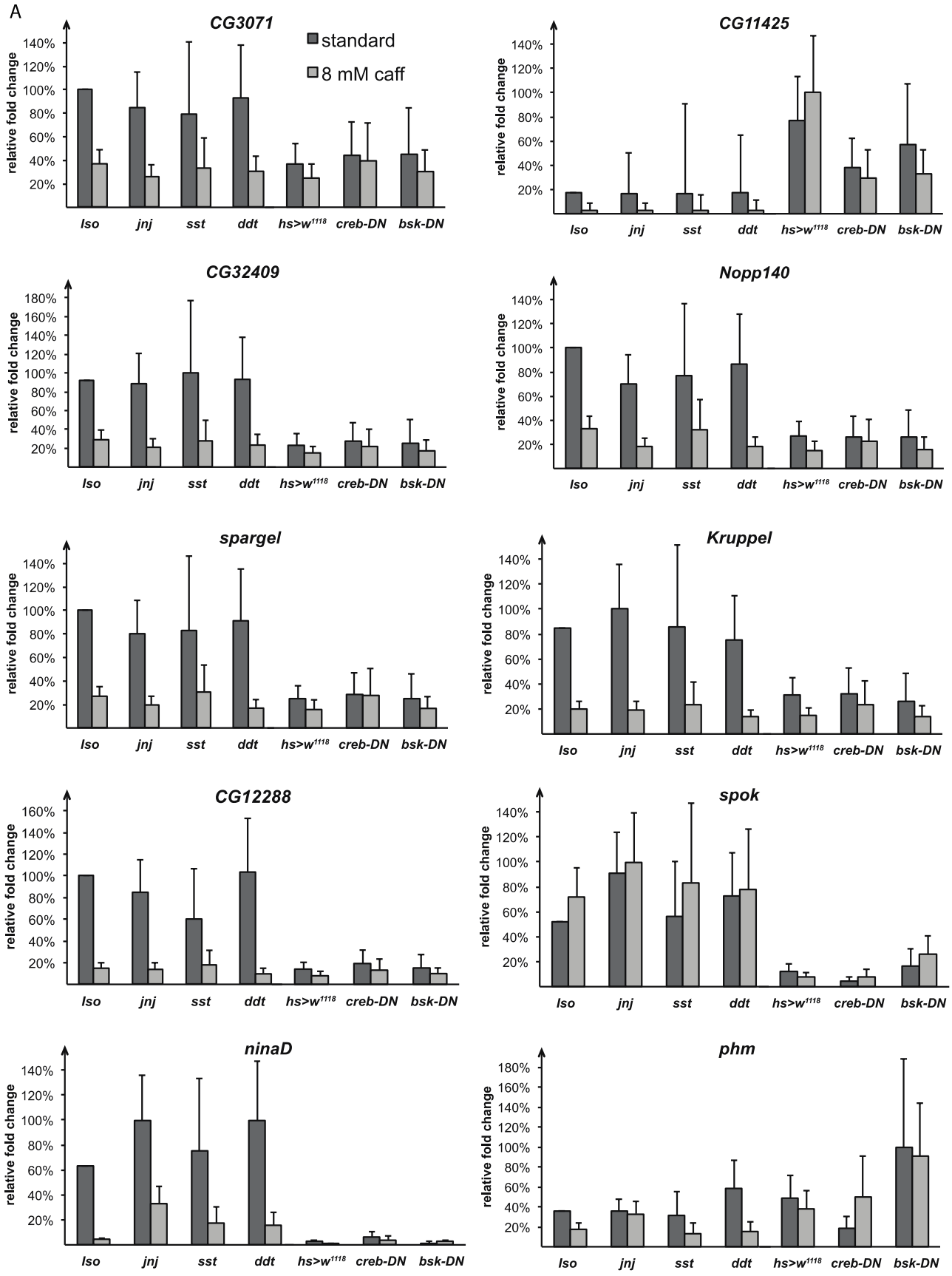
Similarly CREB is activated through phosphorylation by PKA, which in turn is activated by cAMP that was generated upon stimulation of extracellular signal. CREB binds to CRE on the DNA level, and thus activates or represses its target genes. D-jun is autoregulated by AP-1 binding but is also activated by CREB. Caffeine and its metabolite theophylline inhibit cAMP phosphodiesterase, which degrades cAMP (*97-99*), so the effect of cAMP is prolonged. In *Drosophila*, there are two genes encoding the CREB protein family members: *CrebA* and *CrebB-17A (crebB).* Of the two, *crebB* protein shows a higher degree of similarity to mammalian *CREB*. In order to test my hypothesis that caffeine

induces P450 genes through JNK and cAMP pathways, I first examined the viability of dominant negative forms of JNK (i.e. *basket*) and CREB (i.e. *crebB*) (*114*) before testing them for loss of caffeine engendered transcriptional changes in caffeine responsive genes. Ubiquitous expression of *UAS-bsk-DN* and *UAS-crebB*^{cbz} (hereafter cbz) by *Act-Gal4* produced 16-20% of survivors or no survivor respectively.

Thus *heat shock-Gal4* was the driver of choice for *bsk-DN* and *cbz-DN* in 4 hr old L3. Because *bsk-DN* and *cbz-DN* were constructed in a w^{118} background, *heat shock-Gal4* driver crossed to w^{118} was used as the appropriate control for them.

Differentially expressed genes in response to caffeine-treatment were tested in caffeine sensitive *jnj*, *sst* and *ddt* homozygous or hemizygous mutant lines as well as heat shock-driven dominant negative alleles of *creb* and *bsk* (Figure 3.8). Because 8 mM caffeine prompted a more drastic detoxification response than 2 mM caffeine with essential the same trend, 8 mM was used to treat aforementioned mutants and their controls. There is no obvious difference in expression levels between Iso controls and the caffeine sensitive mutants, nor between the *hs>*w^{1118} control animals and the *hs>bsk-DN* or the *hs>creb-DN* lines. For the translation-related genes *CG3071, CG32409, Spargel* and *CG12288*, as well as the top-repressed gene *ninaD* the overall expression in the strains *hs>*w^{1118}, *hs>bsk-DN* and *hs>creb-DN,* which have a w^{118} background, is lower than the *Iso* background, which includes *Iso, jnj, sst* and *ddt*. For the phosphoesterase *CG11425* and the ecdysone biosynthesis enzyme genes *spok* and *phm*, the heat shocked *hs>*w^{1118}, *hs>bsk-DN* and *hs>creb-DN* have a more

undefined pattern, presumably because the impact of the heat shock as a stress perturbes expression profiles (Figure 3.8A). In terms of the P450 genes, *Cyp6a8* expression is reduced in the dominant negative *bsk* and *creb* mutants when compared with controls. This taken together with the previously published finding that there are many predicted binding sites for CREB and AP-1 in the *Cyp6a8* and *Cyp6a2* promoter regions supports the model that PKA and JNK pathways are important for induction of *Cyp6a8* by caffeine. *Cyp6a2* on the other hand was highly induced by heat shock and was unable to respond to caffeine on top of the applied heat shock stress (Figure 3.8B). Reductases *CG9360*, *CG2065* and *CG1441* along side with ABC transporter-encoding genes *CG4822* and *Mdr50* did not respond significantly to caffeine in the w^{1118} background (Figure 3.8C). Heat shock also seems to reduce the response of DUF227-encoding genes and other caffeine induced genes, such as *ref(2)P, Smg5, l(2)efl* and *Dgp-1*, to caffeine treatment (Figure 3.8D and E). Overall, heat shock had a greater effect on gene expression than caffeine for genes tested in *hs>*w^{1118}, *hs>bsk-DN* and *hs>creb-DN*. Meanwhile, caffeine-sensitive *jnj, sst* and *ddt* mutants appear to have a normal detoxification response, suggesting they play no roles in this process.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standard
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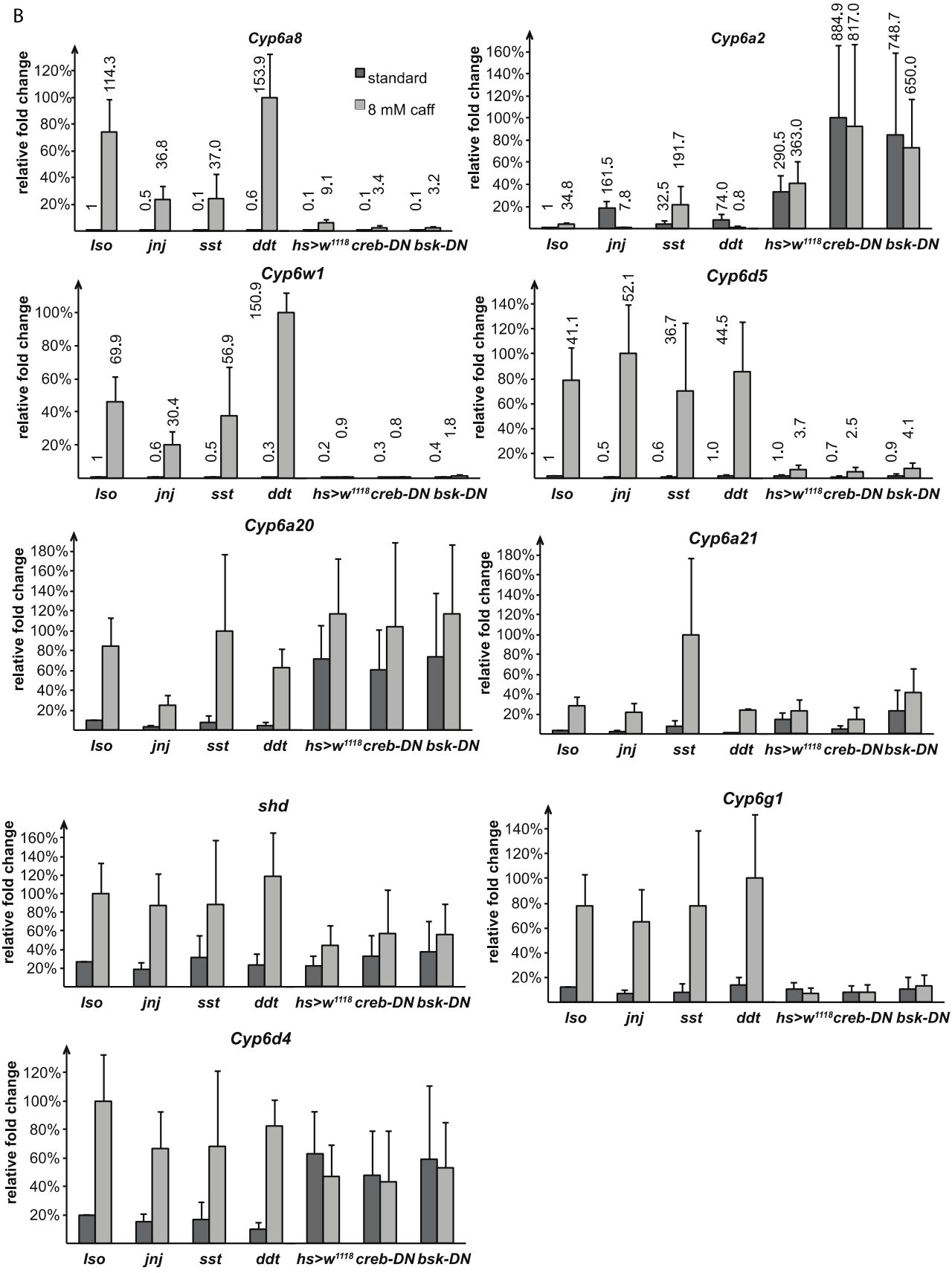
120%
100%
80%
60%
40%
20%
lso
jnj
sst
ddt
hs>w1118
creb-DN
bsk-DN
CG11425
CG32409
180%
160%
Nopp140
spargel
Kruppel
CG12288
spok
ninaD
phm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100%
80%
60%
40%
20%
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36.8
0.1
37.0
0.6
153.9
0.1
9.1
0.1
3.4
0.1
3.2
Iso
jnj
sst
ddt
hs>w1118
creb-DN
bsk-DN
Cyp6a2
160%
140%
120%
100%
80%
60%
40%
20%
1
34.8
161.5
7.8
32.5
191.7
74.0
0.8
290.5
363.0
884.9
817.0
748.7
650.0
Cyp6w1
100%
80%
60%
40%
20%
1
69.9
0.6
30.4
0.5
56.9
0.3
150.9
0.2
0.9
0.3
0.8
0.4
1.8
Cyp6d5
140%
120%
100%
80%
60%
40%
20%
1
41.1
0.5
52.1
0.6
36.7
1.0
44.5
1.0
3.7
0.7
2.5
0.9
4.1
Cyp6a20
180%
160%
140%
120%
100%
80%
60%
40%
20%
Cyp6a21
180%
160%
140%
120%
100%
80%
60%
40%
20%
shd
160%
140%
120%
100%
80%
60%
40%
20%
Cyp6g1
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120%
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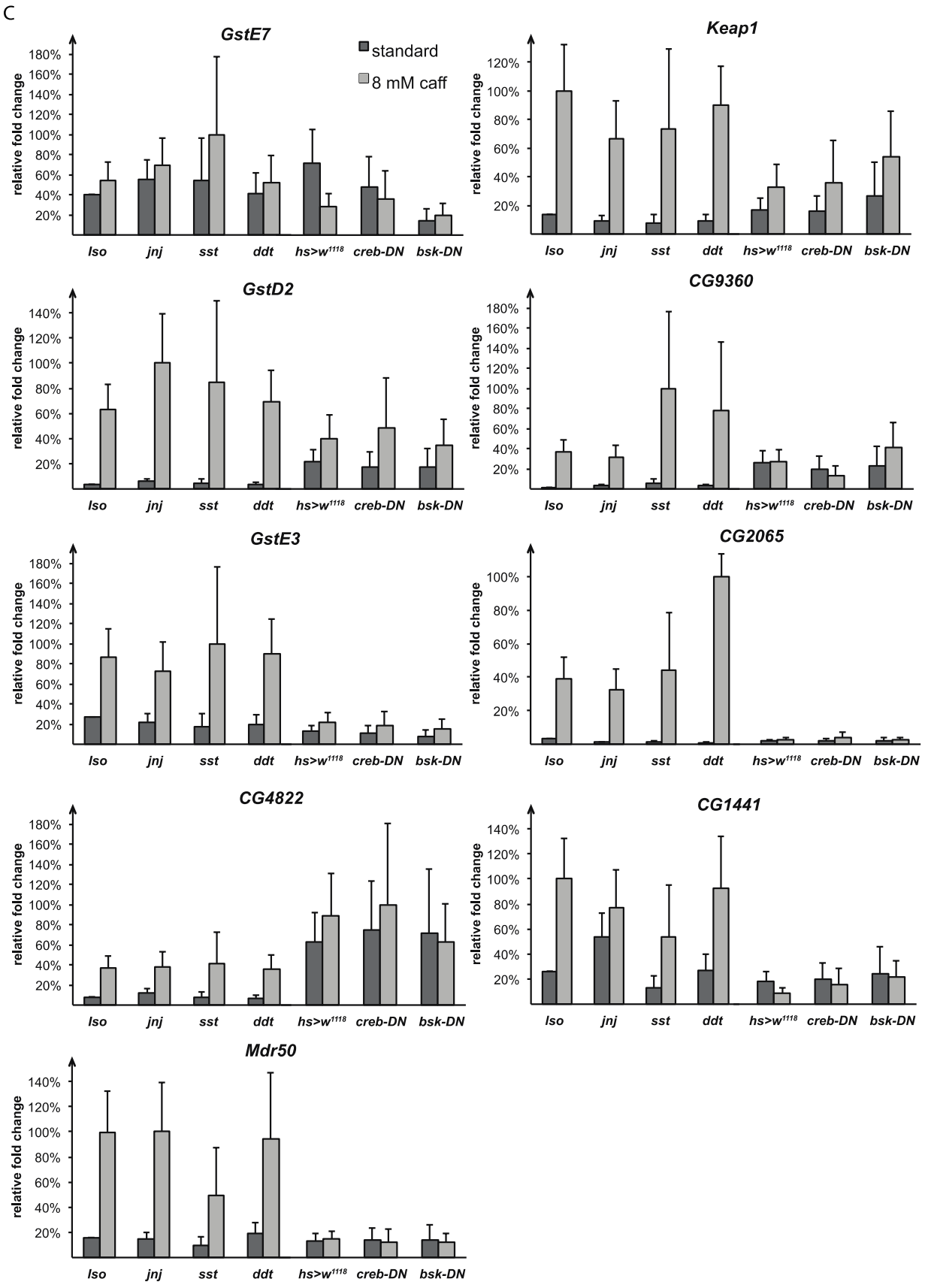
40%
20%
Cyp6d4
120%
100%
80%
60%
40%
20%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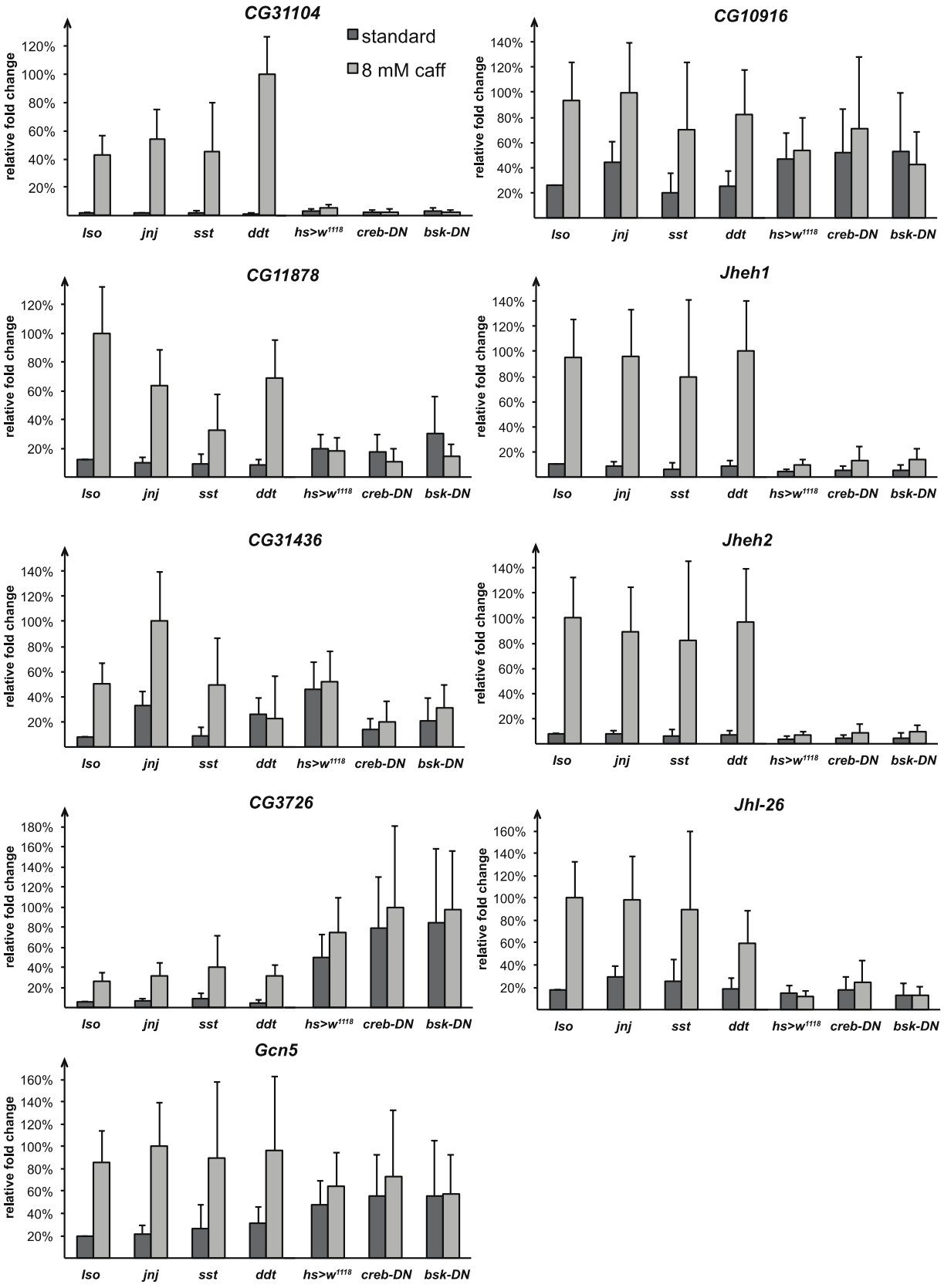
60%
40%
20%
lso
jnj
sst
ddt
hs>w1118
creb-DN
bsk-DN
Keap1
GstD2
CG9360
GstE3
CG2065
CG4822
CG1441
Mdr50

D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80%
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40%
20%
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bsk-DN
CG10916
140%
120%
100%
80%
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160%
140%
Jhl-26
Gcn5

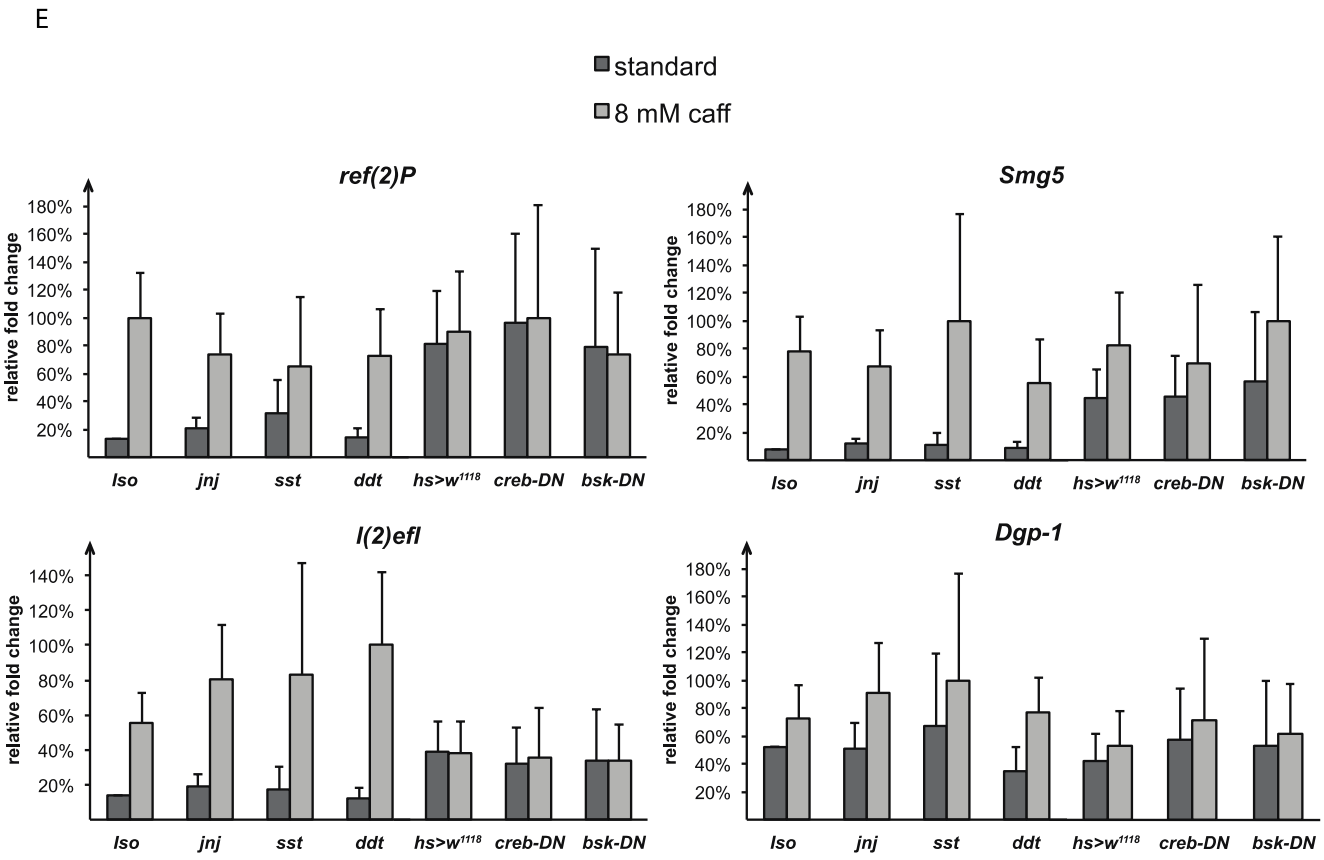


Figure 3.8 Fluidigm qPCR result of differentially expressed genes in caffeine sensitive mutant *jnj*, *sst* and *ddt* as well as dominant negative forms of *creb* and *bsk*, in the presence and absence of caffeine. (A) downregulated genes (B) upregulated P450 genes (C) upregulated GST, reductases, ABC transporter genes and *Keap1* (D) upregulated DUF227, Juvenile hormone response genes and Gcn5 (E) other upregulated genes.

3.3.5 Starvation induction of a P450 gene involved in detoxification: The story of *Cyp6a2*

It is a striking observation that the P450 gene *Cyp6a2* is induced about 100 fold by starvation, since *Cyp6a2* is a well-documented detoxification enzyme that is induced by caffeine *in vitro* and *in vivo*. This is supported by my microarray and the qPCR date, which showed that *Cyp6a2* was induced ~16fold by caffeine. However, there is no report on its induction by starvation alone (Figure 3.9A). I am describing this phenomenon for the first time. This novel observation seems to be dependent on specific genetic background. My caffeine microarray and the high throughput qPCR were performed on *Iso.* Under normal conditions, *Cyp6a2*'s expression is 2fold in w^{1118} comparing with *Iso*. The control P450 gene I choose, *Cyp6a8*, is expressed at a low level in w^{1118} and about 5fold of that in Iso (Figure 3.9B). Heat shock in the w^{1118} background had no effect on *Cyp6a2* but induced *Cyp6a8* and *Cyp6g1* (Figure 3.9C). Thus, the high levels of *Cyp6a2* expression in *hs*>w^{1118}, *hs*>*bsk-DN* and *hs*>*creb-DN* are due to the genetic background, not heat shock stress. Overexpression of dominant negative forms of *creb* and *bsk* induced *Cyp6a2* even further independent of caffeine, which was not seen with *Cyp6a8* (Figure 3.9D and E). Starvation with w^{1118} or another wild type strain *CantonS* did not produce the same elevated level of *Cyp6a2* expression showing that it was an innate feature of the *Iso* genotype. However, using stocks obtained from the Thummel lab that carry 5 copies of the wild type binding site of dNrf2/Maf from the *Cyp6a2* promoter I was able to demonstrate a slight induction of the reporter LacZ (about 1.7fold) upon starvation (Figure 3.9H). This induction

is reversed to 0.7fold in transgenic strains carrying 5 copies of the mutated dNrf2/Maf binding site with a 15 bp deletion that was first characterized in *Met* mutants (*89*). This suggested that the slight induction of *Cyp6a2* by starvation is attribute to the regulation by *dNrf2/Keap1*.

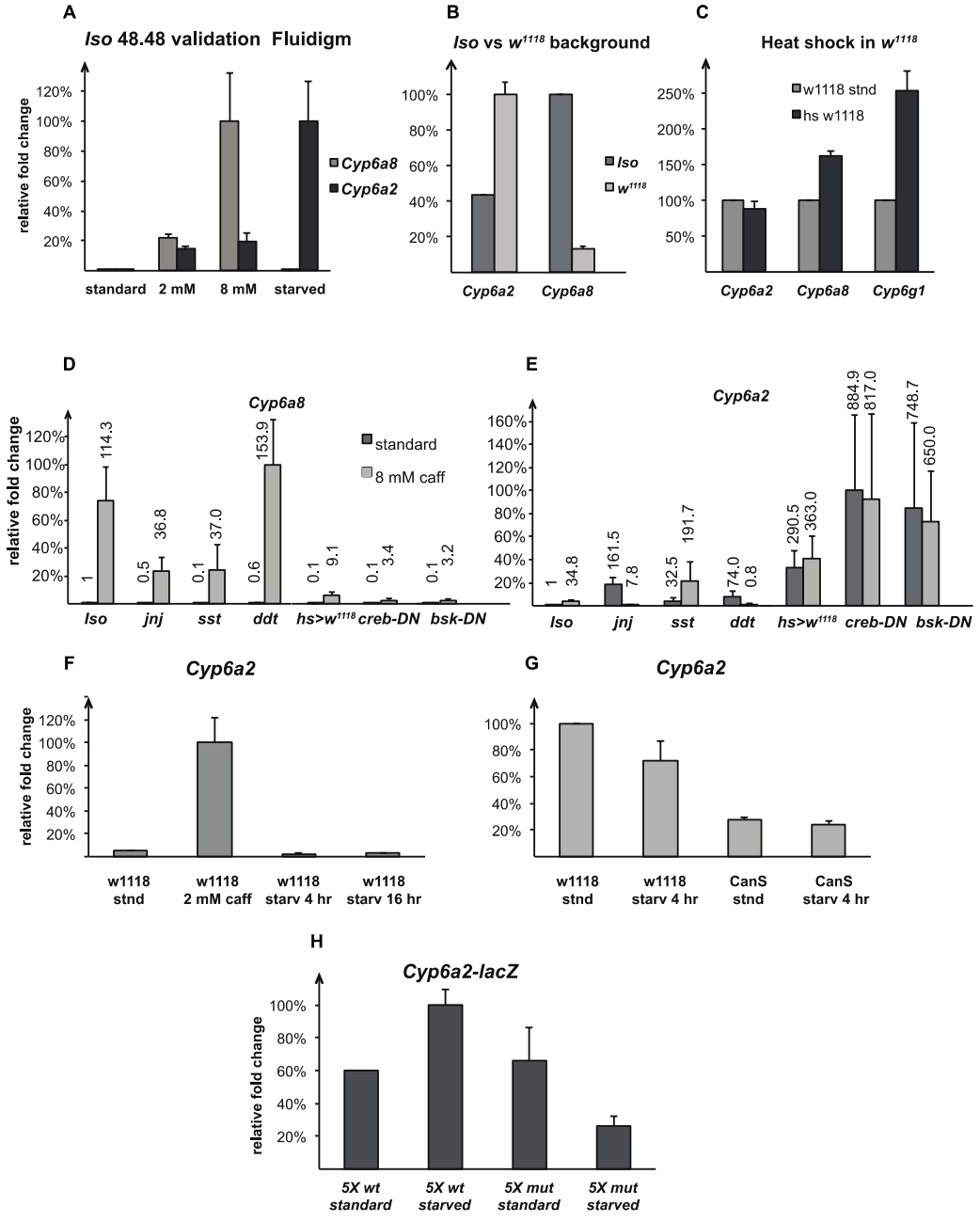


Figure 3.9 Induction of *Cyp6a2* by caffeine is background-independent, while induction of *Cyp6a2* by starvation is background-dependent.

3.3.6 Caffeine-induction of detoxification genes relies on more than one transcription factor

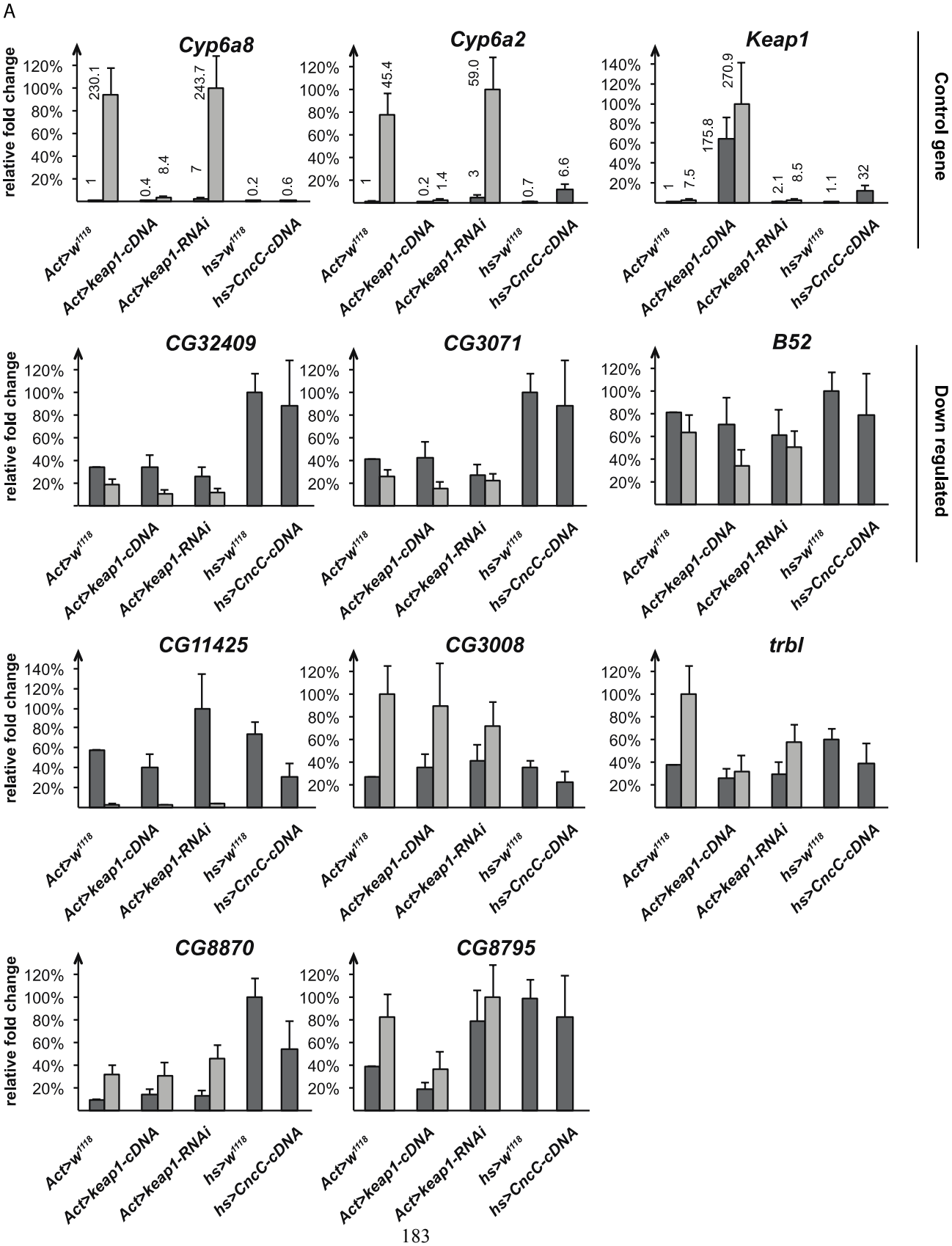
At the time when the caffeine microarray was underway, I was searching for transcription factors that might function as xenobiotic response regulators in *Drosophila*, I first looked at AHR and its binding partner ARNT. Aryl hydrocarbon receptor (AhR) is an important mediator in the metabolic activation and detoxification of carcinogens (*115, 116*). The apparent role of AHR and ARNT bHLH-PAS domain transcription factors in controlling mammalian xenobiotic detoxification prompted me to examine the possibility that this function is conserved in fruit flies. The *Drosophila* Spineless (Ss) protein provides the best match to the bHLH-PAS domain of AHR, and there are six other bHLH-PAS containing proteins in the Drosophila genome. I tesed *Drosophila* bHLH-PAS candidates for regulating xenobiotic responses by knocking down their mRNA level via RNAi and examining whether survival on caffeine media was compromised (Table 3.12). Ubiquitous expression of RNAi aimed at *met*, *sima, clk, tai* and *sim* result in pupal lethality independent of caffeine. There was a slight difference between caffeine-treated and untreated dysfusion-RNAi but it was negligible. Another factor I tested by RNA interference was the fly homolog of HNF4. *Hepatocyte nuclear factor 4* (*HNF4*) is a liver-specific nuclear receptor that controls the expression of a variety of genes, including those involved in cholesterol, fatty acid and glucose metabolism as well as detoxification processes (*117*). I tested HNF4-RNAi in the whole fly by using a ubiquitous actin5C Gal4 driver, but also expressed RNAi in the eye with the eyeless Gal4 driver, since

disturbances in eye patterning are easily scored. There was no noticeable change in fly viability or eye morphology in this case (data not shown), suggesting HNF4 has no roles in the xenobiotic response to caffeine.

In fruit flies, *DHR96* and *dNrf2* are recognized as transcription factors that control the expression of many detoxification genes. I cross-compared the caffeine microarray data with that of DHR96-cDNA and ectopic *dNrf2* microarrays (Figure 3.6). Eighty-four caffeine-induced genes including many detoxification genes were also upregulated by *dNrf2-cDNA* overexpression but only 10 by *DHR96-cDNA*. Moreover, the majority of PB-induced detoxificaton genes in the King-Jones study were *DHR96*-independent. Therefore, I decided to focus on the detoxification genes that are unique to caffeine induction and test them in *dNrf2/Keap1* mutants. The top 10 induced genes unique to caffeine includes four DUF227 genes, two P450 genes, two GSTs, a heat shock protein and a transmembrance transporter, which were all induced > 5fold (Table 3.14). The top 10 repressed gene list unique to caffeine is much like the top 10 list in caffeine microarray, having rRNA processing enzymes, ecdysone biosynthesis enzyme and PA-phosphatase related phosphoesterase among others (Table 3.15). Highly induced gene families unique to caffeine encode the following enriched detoxification enzymes: P450, GST, UGT, redox enzymes, ABC transporters and DUF227 proteins. New GO terms that such as secondary active organic cation transmembrane transporters, Mo-molybdopterin cofactor biosynthesis genes and long chain fatty acid transporter were found in the caffeine unique set (Table 3.16). Unique GO terms for the downregulated caffeine gene set are similar to

downregulated genes in my caffeine microarray: RNP, rRNA and ribosomal assembly genes, genes encoding G-protein beta WD-40 repeats and ABC transporter (Table 3.17).

Normally, dNrf2 is retained in the cytoplasm sequestered by the actin-associated protein Keap1, which also functions as an adapter for dNrf2 proteasomal degradation. Electrophiles and reactive oxygen species disrupt the interaction between Keap1 and dNrf2, causing dNrf2 stabilization and subsequent nuclear translocation. In the nucleus, dNrf2 forms a heterodimer with Maf-s to bind to antioxidant response elements/electrophile response elements in target promoters, and thus up-regulates a wide range of detoxification genes in *Drosophila*. As described above, Keap1 is a negative regulator of dNrf2 function. Defining a set of detoxification genes unique to caffeine is the first step towards understanding the mechanism behind it, because it might give us clues to search for the elusive transcription regulator of xenobiotic response that is not *dNrf2/Keap1*. I expected that if a gene were dependent on dNrf2 for its induction, overexpressing Keap1-cDNA would demolish this induction whereas *Keap1*-RNAi and *dNrf2*-cDNA would induce this gene without drug treatment. Detoxifcation genes I chose from the caffeine unique list were not affected by *dNrf2/Keap1* mutations (Figure 3.10).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Down regulated
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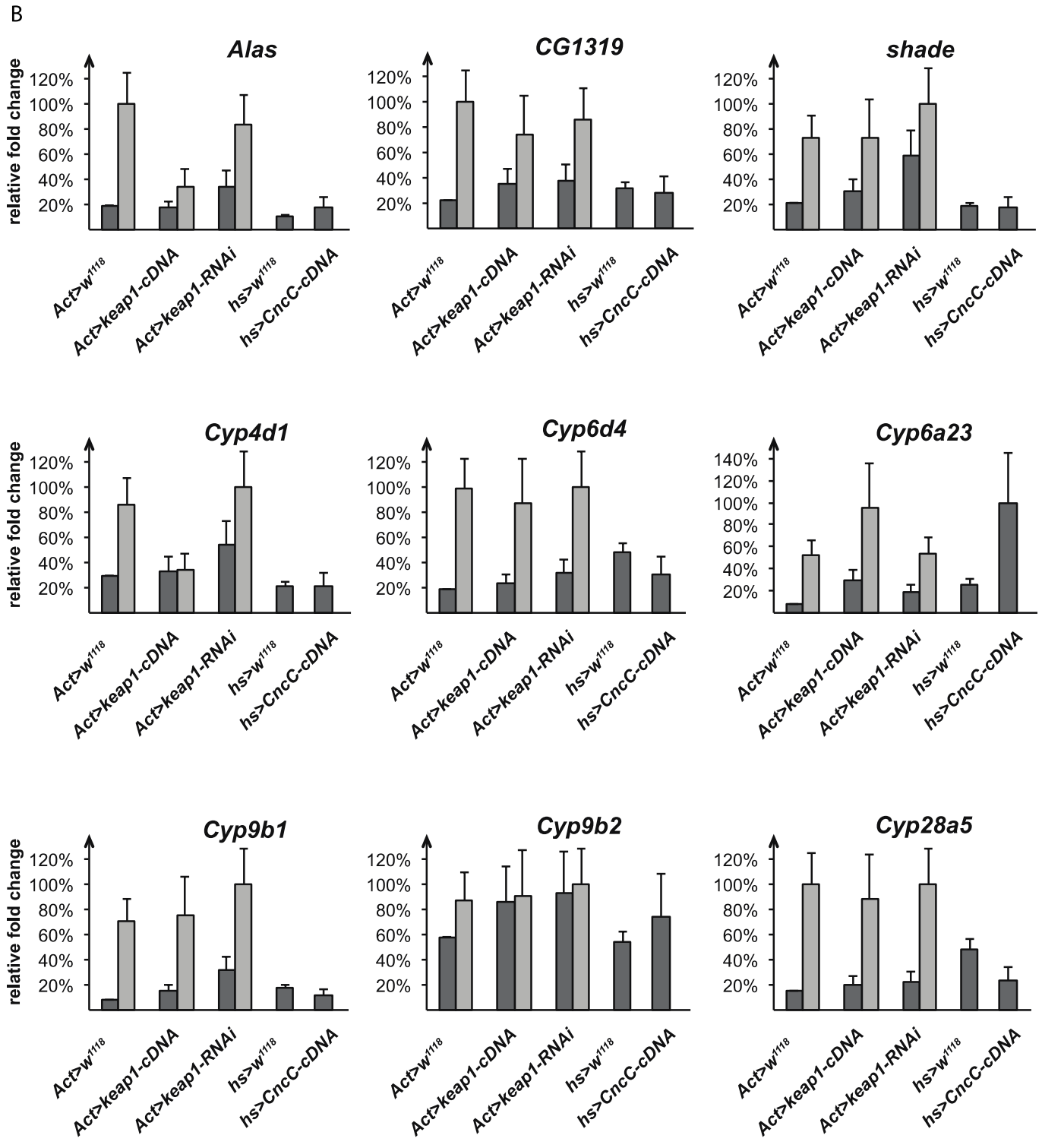
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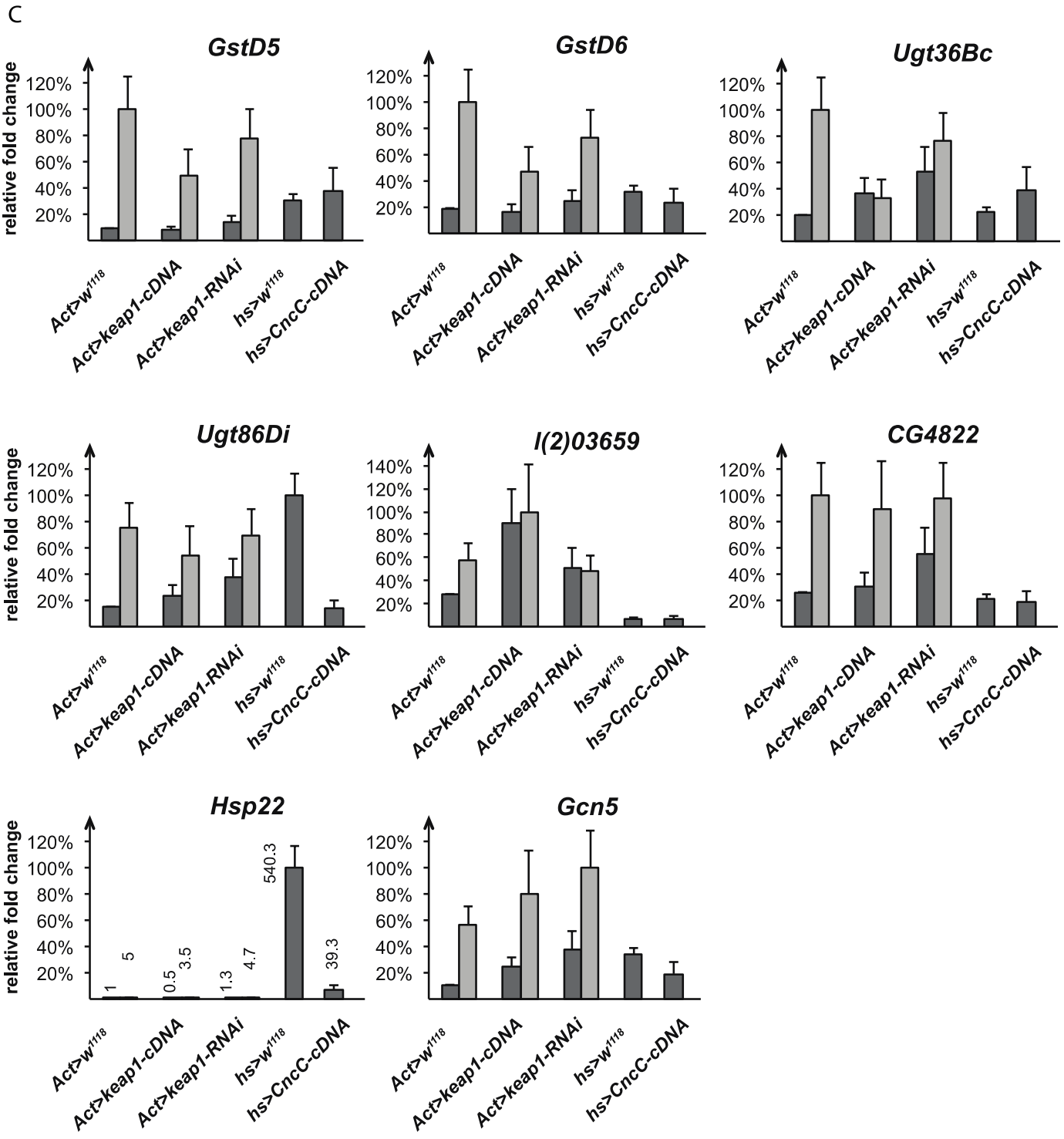
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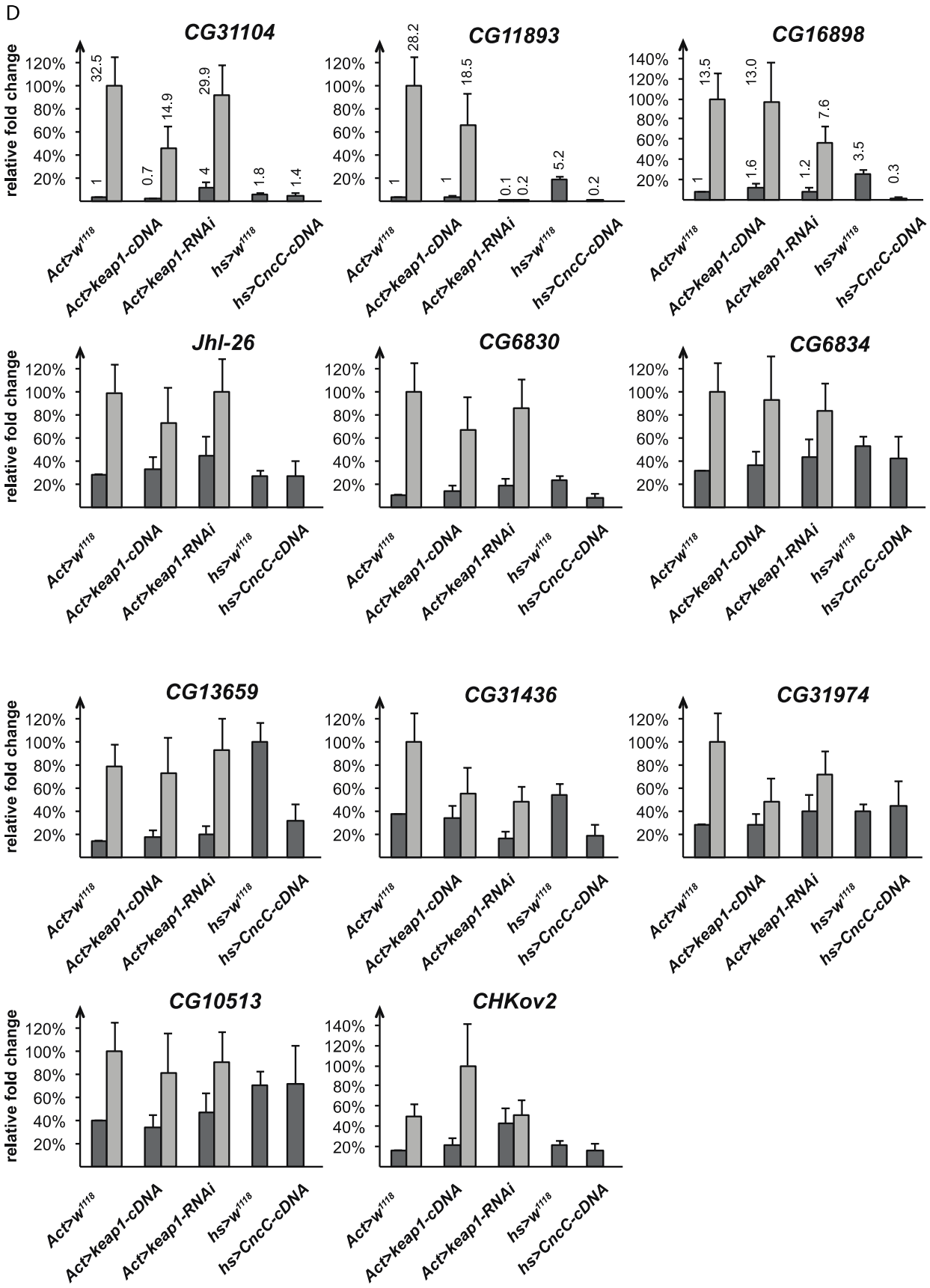
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Act>keap1-cDNA
Act>keap1-RNAi
hs>w1118
hs>CncC-cDNA

Figure 3.10 Fluidigm qPCR analysis of caffeine induced *dNrf2/Keap-1* independent genes. (A) Control genes and caffeine downregulated genes plus genes represent individual caffeine upregulated classes (B) Cytochrome P450 genes (C) GST, UGT, ABC transporters and Heat shock protein, Gcn5 Transcription regulator (D) DUF227 encoding genes.

3.4 Discussion:

3.4.1 Microarray and Fluidigm qPCR validation

Out of the 188 caffeine-induced genes, 79 (42%) are P450, Gst, Ugt and ABC transporter genes, indicating that caffeine is a very powerful xenobiotic. The genome of Drosophila melanogaster contains 99 genes (92 listed in Affymetrix genome probe list) belonging to the cytochrome P450 (P450) family which encode monooxygenases, many of which function in developmental and xenobiotic pathways (*118*). Among the P450s associated with insecticide resistance in *D. melanogaster*, studies have focused on nine genes: *Cyp4e2*, *Cyp6a2, Cyp6a8, Cyp6a9, Cyp6g1, Cyp6w1, Cyp12a4, Cyp12d1* and *Cyp308a1* (*119*). These genes were upregulated in my caffeine microarray with the exception of *Cyp12a4* and *Cyp308a1*. In addition to the well established detox players, 15 additional P450s were induced, four of which displayed more than 10 fold higher transcript levels, demonstrating the importance of P450 genes in detoxification in response to caffeine exposure.

My 8 mM caffeine microarray yielded slightly different results from the FlyBase transcriptome modENCODE mRNA-Seq treatment data. The reason most likely lies in the innate differences of the two platforms microarray vs RNA-Seq as well as different method in analysis and different genetic backgrounds. In general, RNA-Seq is considered superior in detecting low abundance transcripts

and has a broader dynamic range than microarrays, which in comparison tends to allow for the detection of differentially expressed genes with higher fold-changes. Nevertheless, in a report focused on the difference between RNA-Seq and microarray technologies in transcriptome profiling, a comparison of data sets derived from RNA-Seq and Affymetrix platforms using the same set of samples showed a high correlation between gene expression profiles generated by the two platforms (*25, 26, 40, 41, 74, 120-124*). Consistent with this, in the largest comparative study between microarray and RNA-Seq methods to date using The Cancer Genome Atlas (TCGA) data, there were high correlations between expression data obtained from the Affymetrix one-channel microarray and RNA-Seq. Similar to our observation, they also observed that the low abundance genes had poorer correlations between microarray and RNA-Seq data than high abundance genes (23). Overall, the trend and direction of differential expression is the same between the two platforms. Our microarray seemed to offer a more inclusive picture of caffeine-induced transcriptome with a wider range of detoxification gene expression. The modENCODE treatment expression study used a wild type strain Oregon-R whereas in my own caffeine microarray study I was a Isogenized stock having FRT sites on the right arm of the third chromosome in a *white* genetic background. As we see in our lab as well as in others work, the expression of detoxification genes, P450s in particular, are very sensitive to the genetic backgrounds. Our study complements the existing modENCODE treatment expression data, providing more useful information on how *Drosophila* responds molecularly to xenobiotics.

The DUF227 domain is another very important gene ontology class (i.e. protein family due to common protein domain) that was induced by caffeine. The second most highly induced gene in the entire array is CG31104 (36.7fold, p=6.61E-08), a DUF227 domain-containing Choline kinase (CHk) like kinase. In the list of 23 DUF227 genes, many were thought to respond to various xenobiotics (see Table 3.4). *CG6908* and *CG10513* were described as PB-inducible and the former was found in *DHR96*-mediated xenobiotic responses (*125*). *CG6989* and *CG11878* are both induced by alcohol (*56, 126*). *CG11893* is another highly transcribed DUF227 CHk kinase-like gene that was associated with moderate and high DDT resistance (*127, 128*). In the literature, a Transposon-mediated truncated form of DUF227 domain-containing CHk-kinase *CHkov1* was found to confer organophosphate insecticide resistance in *Drosophila* species (*129*) strongly suggesting a role of DUF227 domain-containing kinases in insecticide resistance. In *C. elegans*, low doses of IR induce detoxification genes including 4 DUF227 domain-containing genes (*130*) suggesting that the detox function is conserved. In some instances, Pfam reclassifies DUF227-containing CHk kinases as kinases that target insect hormones (Ecdysteroid kinase) that are responsible for the phosphorylation of ecdysteroids (insect growth and moulting hormones) at C-22, to form physiologically inactive ecdysteroid 22-phosphates (*131*). DUF227 CHk kinases are therefore candidates of a novel class of detoxification genes.

Caffeine negatively affected the expression of some 23 ribosome biogenesis genes according to our microarray. Ribosome biosynthesis is a

multifactorial complex process. In budding yeast, the rRNA and ribosomal biosynthesis regulon contains more than 200 genes that function at various levels of the rRNA and ribosome biogenesis pathways and are co-regulated (Figure 3.11 also see (*132*)). It is plausible that caffeine inhibits a key factor that coordinately regulates RRB genes in *Drosophila melanogaster*. In fact, caffeine is a potent PIKK inhibitor; it is a novel small molecule inhibitor of yeast TORC1, homolog to the *Drosophila* target of rapamycin (Tor) kinase (*133*). In *Drosophila*, insulin and Tor pathways regulate GSK3 beta activity to control Myc stability and determine Myc expression *in vivo*. Transcription factor c-Myc activates ribosomal RNA synthesis by RNA polymerase I in addition to controlling RNA polymerase II- and III-regulated gene transcription which are also crucial for a functional ribosome (*134*).

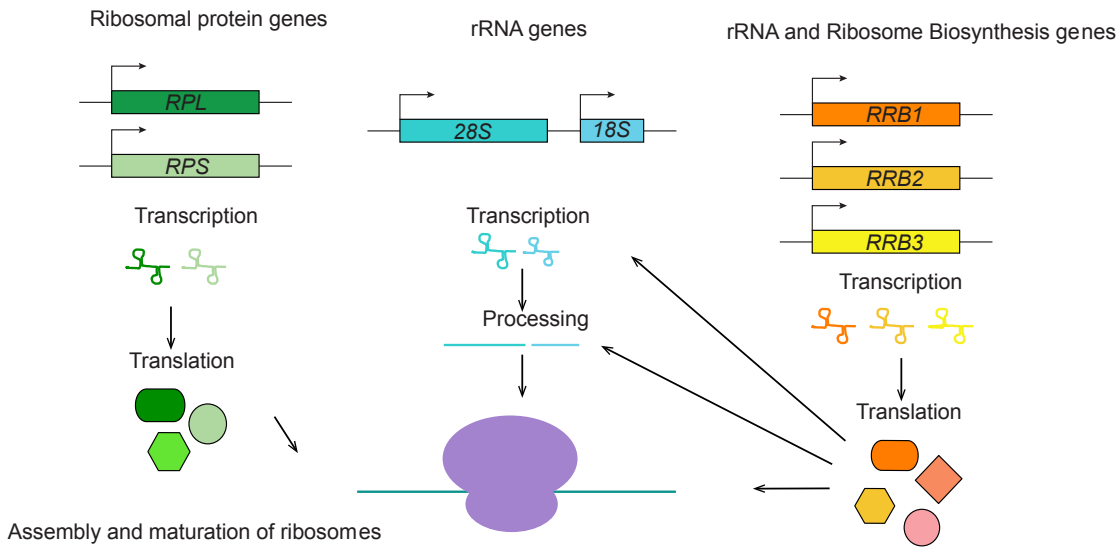

Figure 3.11 Caffeine inhibits the transcription of ribosome biogenesis genes in *Drosophila melanogaster* larvae. Ribosome biogenesis requires the coordinated regulation of three extensive gene networks, including cytoplasmic ribosomal protein genes, rRNA genes and RRB genes. Our microarray showed that caffeine repressed the expression of RRB genes. Figure adapted from

Figure 1 on Dr. Michael McAlear Wesleyan University research web page http://mmcalear.faculty.wesleyan.edu/research/ accessed Apr 22, 2014 and modified.

TIF-IA is the factor mediating growth-dependent control of rRNA synthesis. In mammals, TIF-IA not only interacts with RNA Pol I but growth dependent regulation of RNA polymerase I (Pol I) transcription is also mediated by TIF-IA (*109*). Downregulation of Tif-IA transcripts by caffeine halts transcription from RNA pol 1 promoter, which in turn inhibits rRNA transcription. We provided the first evidence that caffeine inhibits global protein translation by downregulating RRB gene expressions in *Drosophila melanogaster*.

Some of the microarray genes are linked to splicing. Eukaryotic alternative pre-mRNA splicing generates functionally diverse isoforms of proteins from a single gene by mixing and matching adjacent exons. It greatly contributes to the complexity of the eukaryotic proteome. Alternative splicing responds to developmental and environmental cues and is carried out in a development-specific and tissue-specific manner. It is estimated that ~60% of human genes are alternatively spliced; about 50% of human genetic diseases are due to mutations that result in mistakes in alternative splicing choices (*135*). Because of the high complexity of splicesome recruitment and splice site recognition, the regulation of alternative splicing is only partially understood. So far we have learnt that along side of RNA regulatory motifs in the specific pre-mRNA, two classes of protein factors facilitate the process: the splicing repressor heterogeneous nuclear ribonucleoproteins (hnRNP) and splicing enhancers serine/arginine-rich (SR) proteins. Caffeine is shown to induce SR factor SC35 (or SR splicing factor

SRSF2) but repress SRSF3 in both transcript and protein levels in human cell culture thus influence the alternative splicing of their down stream target mRNA (*136, 137*). Caffeine induced SR factor SC35 is required for alternative splicing of tumor suppressor gene *Krüppel-like factor 6* (*KLF6*) (*138*). The SC35 alternatively spliced isoform renders *KLF6* inactive by including a cryptic exon 1a. Caffeine's strong effect on splicing is demonstrated by the fact that other 40 cancer-associated genes (out of 524 genes on a human cancer gene microarray) were found to exhibit caffeine-mediated alternative splicing (*139, 140*). Consistent with that idea, caffeine down regulates the transcript and protein levels of SR factor SRSF3. By doing so tumor suppressor *p53* is alternatively spliced to the *p53β* isoform that promotes cellular senescence. Other known SRFS3 targets were also affected by caffeine in a similar manner (*139*). In our *Drosophila* microarray, we found eleven splicing factors encoding transcripts downregulated by caffeine. These include *hnRNA binding protein 1*, *serine/arginine rich protein 55* as well as the *Drosophila SC35*. All of these genes take part in posttranscriptional regulation of developmental genes or genes that act in reproduction processes. Interestingly some are involved in ribosome biogenesis or the insulin pathway as well, for instance *mod*, *hoip* and *Spargel*. *mod* also has the ability to bind DNA and is a dominant suppressor of PEV that affects higher-order chromatin structures (*140*). Our results showed for the first time a caffeine-mediated down-regulation of more than one splicing factor in flies. The implication of such a result is that caffeine might control various alternative pre-mRNA splicing factors and their downstream targets. Caffeine thus appears to

modulate post-transcriptional regulation of gene expression, and might have a bigger influence on the development and metabolism of the organism than previously thought.

In the 96.96 Fluidigm experiment, the wild type control *Iso* showed that most of the genes I tested were caffeine-responsive (Figure 3.7). Translation-related genes were downregulated by caffeine and starvation, showing caffeine's direct transcriptional effect on the Tor pathway. Additional evidence demonstrating caffeine's involvement in reducing Tor pathway signaling is the fact that ecdysone biosynthetic enzymes Spok and Phm are downregulated. The most strongly downregulated genes are also repressed by starvation except *CG11425*, which encodes a PA-phosphatase-related phosphoesterase. *CG11425* could be a caffeine-specific phosphoresterase that dephosphorylates substrates upon caffeine exposure, but at the moment not much is known about its function or substrate specificity. Quite unexpectedly, expression of Halloween enzyme *spok* and *phm* did not go down with starvation. Spok and Phm are key cytochrome P450 enzymes that are highly enriched in the *Drosophila* prothoracic gland cells (located in the ring gland, a neuroendocrine organ) throughout larval development, and are required for normal ecdysteriod biosynthesis (reviewed by *141*). Tor inhibition in the prothoracic gland caused a delay in ecdysone production at the end of L3, and importantly, a delay in the induction of *phm* expression (*142*). In our qPCR experiment, *phm* was inhibited by 8 mM caffeine but not starvation. In the case of *spok,* neither 8 mM caffeine nor starvation lowered its expression. This inconsistency in ecdysone biosynthesis enzyme

expression could be due to the issue of collection timing. The caffeine 96.96 Fluidigm was based on 4 hr L3 to maintain consistency with the caffeine microarray. After molting, Halloween enzymes expression might fluctuate because they are no longer required to synthesize ecdysone thus the impact of Tor signaling on Halloween gene expression might be reduced or absent. The upregulated *CG3726* is a DNA binding protein with undefined function that was highly induced by starvation. To assess the role of this BTB/POZ homeodomain containing DNA binding protein in starvation response, more work needs to be done.

3.4.2 Starvation induction of *Cyp6a2*

Cyp6a2 expression varies significantly among genotypes that were considered wild type. The reason for a seeming lack of induction by starvation in w^{1118} or *CanS* could be due to the already high expression of endogenous *Cyp6a2*, at least it would be true for w^{1118}. I need to look thoroughly at endogenous expression of *Cyp6a2* in *CanS* in order to substantiate this idea. Heat shock was ruled out as a contributing factor (Figure 3.9C). The *LacZ* reporter qPCR further strengthened the argument albeit a relatively smaller difference in expression changes. This could due to discrepancy in measuring the endogenous gene vs a transgene reporter by qPCR. The *5XWT-lacZ* and *5XMut-lacZ* constructs were injected into w^{1118} strains maintained at BestGene, a company specialized in embryo microinjection to generate *Drosophila* transgenics (*89 supplimentary methods*). The w^{1118} strain used by our lab was obtained from Bloomington Drosophila stock center years ago. Over the years the two stocks of the same

genotype might have accumulated different mutations that became fixed in the population. The effect of genetic background can be further addressed by quantifying *Cyp6a2* expression in the w^{1118} strain from BestGene, I expect to see an expression level lower than our w^{1118} strain, because I think the high level of endogenous *Cyp6a2* expression in our w^{1118} strain masked the caffeine-induced expression increase. Starvation is not traditionally considered a condition that induces P450 enzymes. Nutrient uptake is linked to ecdysone production through modulation of the activity of the TOR pathway (*142*). However, there are cases in vertebrate and insect systems where starvation activated P450 genes. In fatheaded minnows, *Pimephales promelas,* unfed females had an increased level of PXR and *CYP3A4* transcripts, and displayed a more pronounced response to xenobiotics (*143*). The fat body-specific *CYP4C1* expression was highly induced by starvation in decapitated cockroach *B.discoidalis (17)*. Induction of *Cyp6a2* by starvation might be a first example in *D. melanogaster*.

3.4.3 An unique set of detoxification genes induced by caffeine is possibly under the control of a novel transcription regulator of metabolic detoxification of xenobiotics

The *dNrf2* pathway was tested in the DDT-resistant 91-R strain; approximately 20% of the genes that were differentially expressed in the 91-R strain are known *dNrf2* target genes thus *dNrf2* pathway is only partially responsible for the over transcription of detoxification enzymes seen in 91-R(*90*). This raises an interesting possibility that one or more additional transcriptional regulators fulfill the role of xenobiotic detoxification regulation in *D.*

melanogaster. My finding supports that some of the detoxification genes I tested were not dependent on dNrf2/Keap1 pathways for their expression. The Nrf2 overexpression is done with a heatshock driven CncC-cDNA (Nrf2), it is wise to test the expression level of Nrf2 in this system to ensure the expected high level of Nrf2 present. This would show whether the CncC-cDNA worked or not. Although this problem is partially remedied by the indirect evidence that Keap1 expression changed in the hs>CncC-cDNA line assuming Keap1 induction by caffeine (5 fold, as seen in the caffeine microarray) is dependent on Nrf2. Future studies will have to address whether other transcription factors are involved in the insect response to xenobiotics.

Tables

Table 3.2 Highly enriched gene families in upregulated gene set of 8 mM caffeine microarray

Gene family based on InterPro domains or GO terms	**Total gene number in genome**	**Gene number found in array**	***P*-value**
P450	92	25	6.59E-141
Redox	647	32	1.14E-24
GST	45	14	5.29E-101
UGT	38	10	2.09E-60
Transporter	433	13	2.34E-05
ABC transporter	23	2	0.00021
DUF227	41	23	2.76E-234
Stress	62	5	2.22E-08

Eight highly enriched gene ontology terms or protein family based on InterPro domains in caffeine upregulated gene set. The top 5 are known to directly facilitate detoxification of xenobiotics. Stress response is also tied into detox processes.

Table 3.3 Cytochrome P450 genes, GST, UGT and ABC transporters enriched in 8 mM Caffeine microarray with comparison to FlyBase transcriptome modENCODE RNA-Seq treatment data and tissue specificity

Gene Symbol	**Gene Title**	**FC**	***P* -value**	**FlyBase**	**Tissue**
Cyp6a8	Cytochrome P450 related AF5	60.71	5.7E-11	Very high: 824	Malpighian Tubules
Cyp6w1	Cyp6w1	30.91	8.1E-08	Very high: 765	Midgut
Cyp6a2	Cytochrome P450-B1	16.79	8.2E-08	Very high: 544	NID
Cyp4d14	Cyp4d14	10.41	9.1E-07	Moderate high: 49	Midgut
Cyp6d5	Cyp6d5	9.26	1.4E-07	Extremely high: 2421	Fat Body/Midgut
Cyp12d1	Cyp12d1-d/p	8.13	1.6E-04	High: 50-75	Malpighian Tubules
CG6870	Cytochrome B5	7.16	4.0E-06	Moderate: 18	Fat Body
Cyp4p1	Cytochrome P450-4p1	5.67	9.3E-06	Very high: 101	Malpighian Tubules/Midgut/Fat Body
Cyp6a21	Cyp6a21	5.58	7.6E-09	High: 73	Midgut
Cyp12a5	Cyp12a5	5.55	2.1E-04	Very high: 319	Hindgut/Midgut
Cyp9b2	Fly plexin a	5.07	5.8E-08	Very high: 884	Malpighian Tubules/Midgut/Fat Body
Cyp28a5	Cyp28a5	5.03	3.9E-06	Very high: 121	Trachea/Fat Body
Cyp6d4	Cyp6d4	3.68	1.5E-07	Moderate high: 48	Midgut/Fat Body
Cyp6a20	Cyp6a20	3.66	1.8E-04	Low: 10	Hindgut
Cyp4e2	Cytochrome P450-4e2	3.54	4.1E-07	Very high: 468	Fat Body/Malpighian Tubules
Cyp9b1	Cytochrome P450-9b1	3.22	2.9E-05	Moderate high: 48	Fat Body/Hindgut/Midgut
Cyp6a17	Cyp6a17	3.12	1.5E-03	Very high: 148	Malpighian Tubules/Fat Body
Cyp6g1	CYP6-like	3.02	8.7E-03	Very high: 147	Fat Body
Cyp6a14	Cyp6a14	2.99	8.6E-05	Very high: 144	Midgut
Cyp6a23	Cyp6a23	2.51	7.3E-06	High: 64	Midgut/Malpighian Tubules
Cyp6a22	Cytochrome P450 related BF6-2	2.13	3.8E-02	Low: 7	Fat Body/Malpighian Tubules
Cyp4d1	Cytochrome P450 4D1	2.03	4.4E-05	Moderate high: 49	Midgut
shd	shade	2.03	1.7E-04	Moderate high: 39	Malpighian Tubules/Midgut/Hindgut/Fat Body
Cyp4p2	Cyp4p2	2.01	6.2E-03	Moderate high: 44	NID
GstD5	Glutathione S transferase D5	21.31	1.0E-08	Very low: 3	Midgut
GstD2	Glutathione S transferase D2	12.02	1.2E-09	Very high: 816	Hindgut
GstD4	Glutathione S transferase D4	10.67	2.4E-06	Moderate high: 40	Fat Body
GstD7	Glutathione S transferase D7	7.80	4.1E-06	High: 95	Midgut
GstD6	Glutathione S transferase D6	6.34	3.0E-05	Moderate high: 46	Midgut
GstE1	Glutathione-S-transferase	4.78	1.2E-07	Moderate: 23	Trachea/Fat Body/Hindgut
GstD3	Glutathione S transferase D3	4.14	6.0E-07	Very high: 247	Salivary Gland
GstE3	Glutathione S transferase E3	4.13	4.5E-05	Very high: 317	Fat Body

GstD10	Glutathione S transferase D10	3.93	2.8E-07	Very high: 216	Fat Body
GstE7	Glutathione S transferase E7	2.97	4.0E-03	High: 60	Midgut
GstE5	Glutathione S transferase E5	2.74	4.8E-03	Moderate high: 27	Malpighian Tubules/Midgut/Hindgut
GstD9	Glutathione S transferase D9	2.44	9.7E-06	High: 55	Salivary Gland/Fat Body/Malpighian Tubules/Trachea
CG6776	GstO3	2.34	1.9E-05	Very high: 208	Carcass/Fat Body
GstE6	Glutathione S transferase E6	2.17	9.5E-06	Very high: 161	Hindgut
Ugt86Dd	Ugt86Dd	25.25	1.4E-07	Low: 10	Malpighian Tubules
CG5999	CG5999	10.33	2.0E-06	Very high: 257	Malpighian Tubules
Ugt36Bb	Ugt36Bb	9.11	5.7E-08	0	NID
CG9360	CG9360	6.66	1.5E-05	Very low: Midgut	Trachea/Hindgut/Midgut
Ugt36Bc	Ugt36Bc	3.19	3.3E-05	Moderate high: 26	Midgut
CG15661	CG15661	3.14	8.3E-06	Low: 4	NID
Ugt37b1	UDP-glycosyltransferase 37b1	2.25	1.3E-03	Low: 5	Malpighian Tubules
Ugt36Ba	Ugt36Ba	2.14	7.5E-04	Low: 6	Midgut
CG31810	CG31810	2.12	4.2E-05	Low: 7	Salivary Gland
Ugt86Di	Ugt86Di	2.08	4.3E-05	0	CNS
Mdr50	Multi drug resistance 50	8.38	8.8E-09	Moderate high: 47	Midgut/Hindgut
l(2)03659	lethal (2) 03659	2.21	1.5E-03	0	Hindgut
CG4822	CG4822	2.00	1.4E-04	very low: 3	Fat Body

FC:fold change. NID=no informative data. CNS= central nervous system. FlyBase=FlyBase expression data 8 mM caffeine L3 from ref(*144*) Note: Cyp12d1-d and Cyp12d1-p were combined together as one for simplicity. FlyBase expression information was listed with first category and then value. All the expression-enriched tissues were listed as long as they are in the same category (very high expression, high expression, moderate expression, etc.) of the top expressed tissue based on FlyAtlas larval Organ/Tissue Expression.

Table 3.4 List of DUF227 domain encoding genes that are affected by caffeine

Gene Symbol	**Gene Title**	**FC**	***P* -Value**	**Implied function, tissue specificity**
CG31104	CG31104	36.74	6.61E-08	None, Midgut
CG6908	CG6908	21.62	4.49E-07	PB inducible, xenobiotic responds, Malpighian Tubules
CG13659	CG13659	16.59	4.89E-08	None, Midgut
CG16898	CG16898	11.68	2.54E-06	None, Midgut, Hindgut
CG10550	CG10550	7.73	8.29E-08	None, Midgut
CG31288	CG31288	7.26	7.57E-08	None, Midgut
CG6830	CG6830	5.84	5.83E-07	None, Midgut
CG10562	CG10562	5.61	2.71E-06	None, Midgut
CG10553	CG10553	3.77	1.59E-06	None, Malpighian Tubules
CG10560	CG10560	3.70	3.19E-07	None, Malpighian Tubules
JhI-26	JHI protein 26	3.26	3.70E-07	None, Midgut, Malpighian Tubules
CG31975	CG31975	2.88	3.83E-06	None, Midgut, Malpighian Tubules
CG31974	CG31974	2.85	1.03E-06	Eckinase, Midgut
CG6834	CG6834	2.84	1.74E-05	None, Midgut
CG11878	CG11878	2.84	1.45E-04	Alcohol induced, Malpighian Tubules
CG13658	CG13658	2.66	1.76E-05	None, Malpighian Tubules, Midgut
CG31436	CG31436	2.59	3.69E-04	None, Fat Body
CG10513	CG10513	2.49	1.18E-04	PB inducible, Malpighian Tubules
CG9498	CG9498	2.37	2.44E-05	None, Hindgut
CHKov2	CHKov2	2.30	5.18E-04	None, Malpighian Tubules, Trachea
CG11893	CG11893	2.27	1.37E-05	DDT resistance, Malpighian Tubules, Hindgut
CG18765	CG18765	2.26	2.24E-06	None, Midgut
CG13360	CG13360	-2.51	2.17E-03	None, Fat Body

A list of 23 genes containing DUF227 domain in the caffeine upregulated gene set. The top four genes are highly induced by caffeine with very high significance. CG13360 is the only DUF227 containing gene that was downregulated by caffeine at -2.51fold (p=2.17E-3). FC:fold change. Tissue expression data was derived from FlyAtlas Organ/Tissue expression microarray on FlyBase (*144*). Only the tissues with high expression were considered with a difference in expression between listed tissues less then 100fold.

Table 3.5 Highly enriched gene families in downregulated gene set of 8 mM caffeine microarray

Gene family based on InterPro domains or GO terms	**gene number in genome**	**Gene number found in array**	***P*-value**
RNP 1	156	8	2.11E-09
rRNA	42	17	6.42E-179
Ribosomal assembly	40	5	2.46E-16
RNApol 1 transcription regulator	1	1	4.05E-28
Transcription	474	11	0.0002
Nuclear mRNA splicing	72	6	1.44E-12
Negative regulation of RNA splicing	2	1	1.26E-14
Transmembrane transport	433	14	1.79E-08
Chitin	105	4	0.0007

Nine highly enriched gene ontology terms or protein families based on InterPro domains in caffeine downregulated gene set. Three classes are known to directly involved in ribosome biogenesis and control of translation. Level of transcript associated with mRNA splicing process is appeared to be altered

Table 3.6 List of caffeine downregulated genes that function in the regulation of protein translation

Gene Symbol	**Gene Title**	**FC**	***P*-value**	**Function**
CG11837	CG11837	-2.50	2.57E-04	rRNA modification
1628188_at	1628188_at	-2.89	3.36E-04	rRNA processing
Nnp-1	Nnp-1	-2.59	8.32E-04	rRNA processing
l(2)k07824	lethal (2) k07824	-2.36	9.01E-04	rRNA processing
CG13097	CG13097	-2.24	3.76E-04	rRNA processing
CG9799	CG9799	-2.03	2.51E-03	rRNA processing
CG3071	CG3071	-2.64	7.75E-04	rRNA processing
l(2)k09022	CG10805	-1.96	5.11E-04	rRNA processing
CG7637	CG7637	-2.78	5.88E-04	rRNA processing
CG6712	CG6712	-2.35	3.75E-04	rRNA processing
CG5033	CG5033	-2.16	6.68E-04	rRNA processing
CG8545	CG8545	-2.65	7.94E-04	rRNA processing
NHP2	NHP2	-3.19	4.72E-04	rRNA processing
CG8939	CG8939	-2.05	1.44E-03	rRNA processing
nop5	nop5	-2.36	1.25E-03	rRNA processing
CG13096	CG13096	-2.29	1.67E-03	Ribosomal protein L1
CG34316 /// mRpS10	mitochondrial ribosomal protein S10	-3.28	9.77E-04	Translation
CG1381	CG1381	-2.27	1.09E-03	Ribosome biogenesis
Bys	bystin	-1.91	3.11E-04	Ribosome biogenesis
CG11583	CG11583	-2.38	2.30E-04	Ribosome large subunit biogenesis
CG32409	CG32409	-2.51	6.36E-04	Ribosome biogenesis
CG7006	CG7006	-2.70	1.26E-03	Ribosome assembly
Tif-IA	Tif-IA	-1.92	1.33E-03	Regulation of transcription from RNA polymerase I promoter

Twenty-three genes were found in the caffeine downregulated gene set with a known function in the regulation of protein translation. Fourteen genes are associated with generating mature ribosomal RNA. Six genes are essential for ribosome biogenesis. Tif-IA regulates the transcription of RNA Pol I, which in turn regulates rRNA transcription. FC:fold change. Function derived based on gene ontology biological process or InterPro protein domain.

Table 3.7 List of caffeine downregulated genes that function in the regulation of mRNA splicing

Gene Symbol	**Gene Title**	**FC**	***P* -Value**	**Function**
Eap	Exu-associated protein	-2.34	1.62E-03	RNA-binding region RNP-1 (RNA recognition motif)
Mod	modulo	-1.99	1.04E-03	RNA-binding region RNP-1 (RNA recognition motif)
SC35	SC35	-2.13	5.81E-04	RNA-binding region RNP-1 (RNA recognition motif)
CG12288	CG12288	-3.72	8.93E-04	RNA-binding region RNP-1 (RNA recognition motif)
Hrb98DE	hnRNA-binding protein 1	-2.22	6.03E-05	RNA-binding region RNP-1 (RNA recognition motif)
B52	Serine/arginine rich protein 55	-2.02	4.22E-04	RNA-binding region RNP-1 (RNA recognition motif)
Spargel	Spargel	-4.68	1.13E-05	RNA-binding region RNP-1 (RNA recognition motif)
xl6	xl6	-1.96	2.85E-04	RNA-binding region RNP-1 (RNA recognition motif)
hoip /// raps	hoi-polloi /// Partner of Inscuteable	-2.83	3.02E-03	nuclear mRNA splicing, via spliceosome
CG5728	CG5728	-2.10	6.02E-04	nuclear mRNA splicing, via spliceosome
CG13900	CG13900	-2.25	1.17E-03	nuclear mRNA splicing, via spliceosome

Eleven mRNA splicing genes were repressed by caffeine. Eight of them have a RNA recognition motif termed RNA-binding region RNP-1. Three are involved in nuclear mRNA splicing. FC:fold change. Function derived based on gene ontology biological process or InterPro protein domain.

Table 3.8 List of top 50 caffeine-induced genes

Gene Symbol	**Gene Title**	**FC**	***P*-value**	**Function**
Cyp6a8	cytochrome P450 related AF5	60.71	5.73E-11	monooxygenase
CG31104	CG31104	36.74	6.61E-08	Protein Kinase domain DUF227 domain CHk-kinase
Cyp6w1	Cyp6w1	30.91	8.07E-08	monooxygenase
Ugt86Dd	Ugt86Dd	25.25	1.42E-07	glucuronosyltransferase
CG6908	CG6908	21.62	4.49E-07	Protein Kinase domain DUF227 domain CHk-kinase
GstD5	Glutathione S transferase D5	21.31	1.02E-08	glutathione transferase
CG2065	CG2065	17.99	3.95E-07	Short-chain dehydrogenase/reductase
Cyp6a2	cytochrome P450-B1	16.79	8.20E-08	monooxygenase
CG13659	CG13659	16.59	4.89E-08	Protein Kinase domain DUF227 domain CHk-kinase
CG5724	CG5724	15.35	1.18E-07	glucuronosyltransferase
Hsp22 /// Hsp67Bb	heat shock protein hsp22 /// Gene 2	13.41	2.76E-08	heat shock protein
GstD2	Glutathione S transferase D2	12.02	1.24E-09	glutathione transferase
CG16898	CG16898	11.68	2.54E-06	Protein Kinase domain DUF227 domain CHk-kinase
GstD4	Glutathione S transferase D4	10.67	2.44E-06	glutathione transferase
Cyp4d14	Cyp4d14	10.41	9.09E-07	monooxygenase
CG5999	CG5999	10.33	1.96E-06	glucuronosyltransferase
Cyp6d5	Cyp6d5	9.26	1.40E-07	monooxygenase
Ugt36Bb	Ugt36Bb	9.11	5.70E-08	glucuronosyltransferase
Mdr50	Multi drug resistance 50	8.38	8.77E-09	ABC transporter
Cyp12d1	Cyp12d1-d // Cyp12d1-p	8.13	1.58E-04	monooxygenase
GstD7	Glutathione S transferase D7	7.80	4.07E-06	glutathione transferase
CG10550	CG10550	7.73	8.29E-08	Protein Kinase domain DUF227

Gene Symbol	Gene Title	FC	*P*-value	Function
				domain CHk-kinase
CG31288	CG31288	7.26	7.57E-08	Protein Kinase domain DUF227 domain CHk-kinase
CG6870	cytochrome B5	7.16	3.98E-06	Cytochrome b5
Smg5	Smg5	7.02	6.77E-06	nonsense-mediated decay.
CG5953	CG5953	6.89	4.14E-03	lateral inhibition
Jheh2	Juvenile hormone epoxide hydrolase 2	6.73	4.98E-08	epoxide hydrolase
CG9360	CG9360 /// CG9360	6.66	1.49E-05	Short-chain dehydrogenase/reductase
Cyp12d1-d	Cyp12d1-d	6.36	5.89E-04	monooxygenase
GstD6	Glutathione S transferase D6	6.34	3.01E-05	glutathione transferase
CG12868	CG12868	6.22	2.85E-08	*unknown*
Keap1	Keap1	6.06	6.06E-07	inhibitor of dNrf2
CG6830	CG6830	5.84	5.83E-07	Protein Kinase domain DUF227 domain CHk-kinase
Cyp4p1	Cytochrome P450-4p1	5.67	9.34E-06	monooxygenase
CG10562	CG10562	5.61	2.71E-06	Protein Kinase domain DUF227 domain CHk-kinase
Cyp6a21	Cyp6a21	5.58	7.56E-09	monooxygenase
Cyp12a5	Cyp12a5	5.55	2.05E-04	monooxygenase
CG17751	CG17751	5.36	1.74E-05	secondary active organic cation transmembrane transporter
CG1882	CG1882	5.28	7.04E-08	epoxide hydrolase
Cyp9b2	fly plexin a	5.07	5.84E-08	monooxygenase
Cyp28a5	Cyp28a5	5.03	3.89E-06	monooxygenase
Jheh1	JH-epoxide hydrolase	4.95	4.26E-08	epoxide hydrolase
CG7224	Starvation-upregulated protein	4.89	1.65E-03	Protein of unknown function DUF1674
GstE1	glutathione-S-transferase	4.78	1.16E-07	glutathione transferase
CG32237	CG32237	4.62	9.31E-06	GYR motif; YLP motif
Out	outsiders	4.54	3.28E-06	monocarboxylic acid transmembrane transporter

Gene Symbol	**Gene Title**	**FC**	***P*-value**	**Function**
Smvt	Sodium-dependent multivitamin transporter	4.26	5.44E-07	Sodium-dependent multivitamin transporter
GstD3	Glutathione S transferase D3	4.14	5.95E-07	glutathione transferase
GstE3	Glutathione S transferase E3	4.13	4.53E-05	glutathione transferase
Acox57D-p	acyl co-enzyme A oxidase	3.31	1.26E-06	acyl-CoA dehydrogenase

Italic indicates an unknown function. Gene symbols and gene titles are adopted from *Drosophila*_2 na30 annotation file provided on Affymetrix website (accessed 2009) and have been updated using flybase (Sep 2013). FC:fold change. *P*-values are highly significant. Function derived based on gene ontology biological process or InterPro protein domain.

Table 3.9 List of top 50 caffeine repressed genes

Gene Symbol	Gene Title	FC	*P*-value	Function
CG15155	CG15155	-12.40	5.69E-06	GCN5-related N-acetyltransferase
ninaD	neither inactivation nor afterpotential D	-8.35	9.01E-06	scavenger receptor activity
Kr	Kruppel	-6.26	4.10E-04	negative regulator of transcription
CG8129	CG8129	-5.48	6.11E-06	L-threonine ammonia-lyase
CG30031 /// CG4269	CG30031 /// CG4269	-5.14	2.82E-03	serine-type endopeptidase
Spargel	Spargel	-4.68	1.13E-05	negative regulation of insulin receptor signaling pathway
Lsp1alpha	Larval serum protein 1 alpha	-4.42	7.71E-04	nutrient reservoir activity
CG11425	CG11425	-4.30	3.81E-04	PA-phosphatase related phosphoesterase
Spok	spookier	-4.24	1.55E-03	monooxygenase
CG10505 /// DmirCG10505	CG10505	-3.99	2.79E-05	ABC transporter
CG8745	CG8745	-3.88	1.05E-05	Aminotransferase class-II
CG12288	CG12288	-3.72	8.93E-04	RNA-binding region RNP-1 (RNA recognition motif)
CG9826	CG9826	-3.62	4.85E-04	transmembrane transport
Nop56	Nop56	-3.60	7.62E-05	Pre-mRNA processing ribonucleoprotein
Nopp140	Nopp140	-3.58	5.73E-05	Treacle-like, Treacher Collins Syndrome
CG1774	CG1774	-3.51	7.74E-05	*Unknown*
CG34316 /// mRpS10	mitochondrial ribosomal protein S10	-3.28	9.77E-04	Haemolymph juvenile hormone binding
CG9701	CG9701	-3.26	3.82E-05	Glycoside hydrolase
CG6372	CG6372	-3.20	1.05E-03	Peptidase M17
NHP2	NHP2	-3.19	4.72E-04	rRNA processing
Ocn	ocnus	-3.10	2.22E-03	testes specific Janus/Ocnus.
CG15534	CG15534	-3.05	5.98E-05	Ribosomal L11 methyltransferase
Art3	Arginine	-2.99	5.73E-04	Ribosomal L11 methyltransferase

Gene Symbol	Gene Title	FC	*P*-value	Function
	methyltransferase 3			
Cpr51A	CG10112	-2.94	6.72E-04	Insect cuticle protein
1628188_at	1628188_at	-2.89	3.36E-04	ribonucleoprotein complex
CAH2	Carbonic anhydrase 2	-2.85	1.79E-05	carbonate dehydratase
obst-A	CG17052	-2.85	1.81E-05	Chitin binding Peritrophin-A
hoip /// raps	hoi-polloi /// Partner of Inscuteable	-2.83	3.02E-03	Ribosomal protein L7Ae/L30e/S12e/Gadd45
CG7637	CG7637	-2.78	5.88E-04	rRNA processing
CG8083	CG8083	-2.78	2.31E-04	Na+ dependent nucleoside transporter
CG7006	CG7006	-2.70	1.26E-03	Ribosome assembly
CG8483	CG8483	-2.68	6.47E-05	Allergen V5/Tpx-1-related
CG9920	CG9920	-2.65	3.10E-03	Chaperonin Cpn10
CG8545	CG8545	-2.65	7.94E-04	rRNA processing
CG3071	CG3071	-2.64	7.75E-04	rRNA processing
1630661_at	1630661_at	-2.64	3.34E-03	*Unknown*
CG3348	CG3348	-2.62	1.12E-03	Chitin binding
1635691_at	1635691_at	-2.60	1.83E-05	Transcription factor
Nnp-1	Nnp-1	-2.59	8.32E-04	rRNA processing
Hsp83	Enhancer of seven in absentia 2	-2.57	2.38E-04	Heat shock protein
CG12699	CG12699	-2.55	1.16E-03	*Unknown*
Lsd-1	Lipid storage droplet-1	-2.53	1.95E-03	lipid transport
CG13360	CG13360	-2.51	2.17E-03	Protein of unknown function DUF227
CG32409	CG32409	-2.51	6.36E-04	Ribosomal biogenesis
CG11837	CG11837	-2.50	2.57E-04	rRNA modification
CG12785	Maternal transcript 89Ba	-2.49	9.24E-04	rRNA processing
Adgf-A	Adenosine deaminase-related growth factor A	-2.48	9.30E-05	Adenosine/AMP deaminase-related growth factor
CG2121	CG2121	-2.48	8.20E-04	Ion channel regulatory protein,

Gene Symbol	Gene Title	FC	*P*-value	Function
				UNC-93
CG11180	CG11180	-2.40	1.16E-04	RNA binding D111/G-patch domain
CG6854	CG6854	-2.39	2.75E-04	Negative regulator of transcription

Italic indicates function is unknown. Gene symbols and gene titles are adopted from *Drosophila*_2 na30 annotation file provided on Affymetrix website (accessed 2009) and have been updated using flybase (Sep 2013). FC,fold change. P-values are highly significant. Function derived based on gene ontology biological process or InterPro protein domain.

Table 3.10 List of genes tested in 48.48 Fluidigm qPCR

Gene symbol	Gene title	FC	*P* –value	Gene ontology
CG11583	CG11583	-2.4	2.30E-04	Brix domain
Ppan	Peter Pan	-2.3	7.84E-04	Brix domain
CG3071	CG3071	-2.6	7.75E-04	G-protein beta WD-40 repeat
CG7845	CG7845	-2.2	4.62E-04	G-protein beta WD-40 repeat
CG30349	CG30349	-2.1	6.88E-04	G-protein beta WD-40 repeat
CG15155	CG15155	-12.4	5.69E-06	GCN5-related N-acetyltransferase
CG15155		-12.4	5.69E-06	GCN5-related N-acetyltransferase
CG34316 /// mRpS10	mitochondrial ribosomal protein S10	-3.3	9.77E-04	mitochondrial ribosomal protein S10
CG11425	CG11425	-4.3	3.81E-04	PA-phosphatase related phosphoesterase
CG6372	CG6372	-3.2	1.05E-03	Peptidase M17
Nop56	Nop56	-3.6	7.62E-05	Pre-mRNA processing ribonucleoprotein
CG32409	CG32409	-2.5	6.36E-04	Ribosomal biogenesis regulatory protein
NHP2	NHP2	-3.2	4.72E-04	Ribosomal protein L7Ae
hoip /// raps	hoi-polloi /// Partner of Inscuteable	-2.8	3.02E-03	Ribosomal protein L7Ae
B52	Serine/arginine rich protein 55	-2.0	4.22E-04	RNA-binding region RNP-1 (RNA recognition motif)
Spargel	Spargel	-4.7	1.13E-05	RNA-binding region RNP-1 (RNA recognition motif)
CG12288	CG12288	-3.7	8.93E-04	RNA-binding region RNP-1 (RNA recognition motif)
ninaD	neither inactivation nor afterpotential D	-8.4	9.01E-06	Scavenger receptor activity
Nopp140	Nopp140	-3.6	5.73E-05	SRP40
Kr	Kruppel	-6.3	4.10E-04	Zn-finger, C2H2 type TF
dm	diminutive	-2.2	1.73E-03	Basic helix-loop-helix dimerization domain bHLH

Thor	insulin-stimulated eIF-4E binding protein	2.4	3.15E-02	Eukaryotic translation initiation factor 4E binding
Cyp6a8	cytochrome P450 related AF5	60.7	5.73E-11	Cytochrome P450
Cyp6a21	Cyp6a21	5.6	7.56E-09	Cytochrome P450
Cyp12d1-d /// Cyp12d1-p	Cyp12d1-d /// Cyp12d1-p	8.1	1.58E-04	Cytochrome P450
Cyp6a2	cytochrome P450-B1	16.8	8.20E-08	Cytochrome P450
Inr	Insulin recepter	2.29	1.91E-02	Insulin receptor complex
GstD2	Glutathione S transferase D2	12.0	1.24E-09	Glutathione S-transferase
GstD7	Glutathione S transferase D7	7.8	4.07E-06	Glutathione S-transferase
GstE3	Glutathione S transferase E3	4.1	4.53E-05	Glutathione S-transferase
CG9360 /// DsimCG9360	CG9360 /// CG9360	6.7	1.49E-05	Short-chain dehydrogenase/reductase
CG31104	CG31104	36.7	6.61E-08	Protein of unknown function DUF227
CG11878	DUF227-2.8	2.8	1.45E-04	Protein of unknown function DUF227
CG31436	DUF227-low	2.6	3.69E-04	Protein of unknown function DUF227
CG3726	CG3726	2.4	8.78E-03	BTB/POZ domain
ref(2)P	refractaire	3.0	7.87E-04	Octicosapeptide/Phox/Bem1p
Pcaf	Pcaf	2.1	4.95E-06	GCN5-related N-acetyltransferase
l(2)03659	ABC transporter	2.2	1.54E-03	ABC transporter
CG4822	ABC transporter	2.0	1.40E-04	ABC transporter
Mdr50	Multiple drug resistant	8.3	8.77E-09	ABC transporter

Genes tested in four conditions with the Fluidigm 48.48 high throughput qPCR assay. *CG15155* was tested twice with two different primer-probe combinations and thus took two spots on the chip. FC:fold change.

Table 3.11 List of genes tested with caffeine 96.96 Fluidigm qPCR

Gene symbol	Gene title	FC	*P* -value	Function
CG3071	CG3071	-2.6	7.75E-04	G-protein beta WD-40 repeat, vesicle mediated transport, rRNA processing
CG11425	CG11425	-4.30	3.81E-04	PA-phosphatase related phosphoesterase
CG32409	CG32409	-2.51	6.36E-04	Ribosomal biogenesis regulatory protein
Spargel	Spargel	-4.7	1.13E-05	RNA-binding region RNP-1 (RNA recognition motif)
CG12288	CG12288	-3.7	8.93E-04	RNA-binding region RNP-1 (RNA recognition motif)
ninaD	neither inactivation nor afterpotential D	-8.4	9.01E-06	Scavenger receptor activity, CD36 antigen
Nopp140	Nopp140	-3.6	5.73E-05	Neurogenesis, Treacle-like, Treacher Collins Syndrome
Kr	Krüppel	-6.26	4.10E-04	Zn-finger, C2H2 type TF
spok	Spookier	-4.24	1.55E-03	Ecdysone biosynthetic process
phm	Phantom	-2.30	7.81E-03	Ecdysone biosynthetic process
Cyp6a8	Cytochrome P450 related AF5	60.7	5.73E-11	Cytochrome P450
Cyp6a21	Cyp6a21	5.6	7.56E-09	Cytochrome P450
GstE7	Glutathione S transferase E7	3.0	4.00E-03	Glutathione S-transferase
Cyp6a2	Cytochrome P450-B1	16.8	8.20E-08	Cytochrome P450
GstD2	Glutathione S transferase D2	12.0	1.24E-09	Glutathione S-transferase
GstE3	Glutathione S transferase E3	4.13	4.53E-05	Glutathione S-transferase
CG9360	CG9360 /// CG9360	6.66	1.49E-05	Short-chain dehydrogenase/reductase
CG31104	CG31104	36.74	6.61E-08	Protein of unknown function DUF227
CG11878	DUF227-2.8	2.84	1.45E-04	Protein of unknown function DUF227
CG31436	DUF227-low	2.59	3.69E-04	Protein of unknown function DUF227
CG3726	CG3726	2.4	8.78E-03	BTB/POZ domain, DNA binding
ref(2)P	refractaire	3.02	7.87E-04	Viral infectious cycle
Pcaf	Pcaf	2.07	4.95E-06	GCN5-related N-acetyltransferase
CG4822	ABC transporter	2.00	1.40E-04	ABC transporter
Mdr50	Multiple drug resistant	8.38	8.77E-09	ABC transporter
Smg5	Smg5	7.02	6.77E-06	Nonsense-mediated decay
Jheh2	Juvenile hormone epoxide hydrolase 2	6.73	4.98E-08	Juvenile hormone epoxide hydrolase 2
shd	shade	2.03	1.69E-04	Cytochrome P450
Cyp6a20	Cyp6a20	3.66	1.78E-04	Cytochrome P450
Cyp6d5	Cyp6d5	9.26	1.40E-07	Cytochrome P450

Cyp6g1	CYP6-like	3.02	8.71E-03	Cytochrome P450 response to insecticid
Cyp6d4	Cyp6d4	3.68	1.49E-07	Cytochrome P450
CG10916	CG10916	2.63	5.35E-06	Zn-finger, RING
l(2)efl	lethal (2) essential for life	2.94	1.94E-03	Heat shock protein Hsp20
CG2065	CG2065	17.99	3.95E-07	Short-chain dehydrogenase/reductase SDR
JhI-26	Juvenile hormone-inducible protein 26	3.26	3.70E-07	Juvenile hormone-inducible protein 26
Dgp-1	Dgp-1	2.77	1.49E-05	Elongation factor Tu
CG1441	CG1441	2.57	8.02E-06	Male sterility protein, Fatty acyl-CoA reductase
Keap1	Keap1	6.06	6.06E-07	BTB/POZ domain, Kelch repeats, inhibitor of dNrf2
Jheh1	JH-epoxide hydrolase	4.95	4.26E-08	JH-epoxide hydrolase
Cyp6w1	Cyp6w1	30.91	8.07E-08	Cytochrome P450

FC:fold change. Function derived from gene ontology biological process and/or InterPro protein domain

Table 3.12 Ubiquitous knockdown of annotated *Drosophila melanogaster* bHLH-Pas genes testing for caffeine sensitized lethality.

Actin5C-Gal4>bHLH-PAS RNAi	**Molecular function**	***Ratio on 0 mM caffeine**	***Ratio on 2 mM caffeine**	**Notes**
Methoprene-tolerant	Transcription factor, juvenile hormone binding	0	0	Early pupal lethal, no adult structure
Germ cell expressed bHLH-PAS	Transcription factor	1	1.2	Phenotypically normal
Similar	Transcription factor	0	0	Pupal lethal
Tango	Transcription activator, protein heterodimerization activity	0.9	2.4	Phenotypically normal
Clock (104507)	Circadian rhythm regulator Transcription activator	0	0	Late pupal lethal, adult structures visible and normal
Clock (107575)	Circadian rhythm regulator Transcription activator	0	0	Late pupal lethal, adult structures visible and normal
Spineless	Transcription factor, protein heterodimerization activity	0	0	Phenotypically normal
Dysfusion	Transcription factor	0.27	0.15	Phenotypically normal
Cycle	Circadian regulation of gene expression	1.73	1.25	Phenotypically normal
Taiman	Steroid hormone receptor coactivator	0	0	Larval lethal
Single-minded	Transcription repressor and activator activity	0	0	Pupal lethal
Single-minded	Transcription repressor and activator activity	0	0	Pupal lethal

Actin5C-Gal4/CyO is crossed to each of the bHLH-PAS RNAi lines. The number of non-Cy flies (Act5C>RNAi) and Cy flies were scored. Ratio is calculated as number of non-Cy RNAi adults/total number of adults to number of balancer carrying Cy adults/ total number of adults.

Table 3.13 List of genes tested in *dNrf2/Keap1* mutants with 96.96 Fluidigm qPCR

Gene symbol	**Gene title**	**FC**	***P* -value**	**Function**
Cyp6a8	cytochrome P450 related AF5	60.71	5.73E-11	cytochrome P450
Cyp6a2	cytochrome P450-B1	16.79	8.20E-08	cytochrome P450
keap1	Keap1	6.06	6.06E-07	BTB/POZ domain, Kelch repeats, inhibitor of dNrf2
Pcaf	Pcaf	2.07	1.70E-02	GCN5-related N-acetyltransferase
CG30431	CG30431	2.46	2.69E-03	Zn-finger, C2H2 type
CG8870	CG8870	2.55	1.64E-04	Peptidase S1
Hsp22	heat shock protein hsp22	13.41	2.76E-08	response to stress
Alas	Aminolevulinate synthase	3.88	5.08E-07	heme biosynthetic process
CG1319	CG1319	2.18	3.92E-06	Ferredoxin,iron ion binding
Cyp4d1	Cytochrome P450 4D1	2.03	4.42E-05	cytochrome P450
Cyp6a23	Cyp6a23	2.51	7.31E-06	cytochrome P450
Cyp6d4	Cyp6d4	3.68	1.49E-07	cytochrome P450
Cyp9b1	Cytochrome P450-9b1	3.22	2.86E-05	cytochrome P450
Cyp9b2	fly plexin a	5.07	5.84E-08	cytochrome P450
Cyp28a5	Cyp28a5	5.03	3.89E-06	cytochrome P450
shade	Shade	2.03	1.69E-04	cytochrome P450
l(2)03659	Hsp related	2.21	1.54E-03	ABC transporter
CG4822	CG4822	2.00	1.40E-04	ABC transporter
Ugt36Bc	Ugt36Bc	3.19	3.33E-05	glucuronosyltransferase
Ugt86Di	Ugt86Di	2.08	4.28E-05	glucuronosyltransferase
CG15661	CG15661	3.14	8.26E-06	glucuronosyltransferase
GstD5	Glutathione S transferase D5	21.31	1.02E-08	glutathione transferase
GstD6	Glutathione S transferase D6	6.34	3.01E-05	glutathione transferase
Jhl-26	Juvenile hormone-inducible protein 26	3.26	3.70E-07	Protein of unknown function DUF227
CG6830	CG6830	5.84	5.83E-07	Protein of unknown function DUF227
CG6834	CG6834	2.84	1.74E-05	Protein of unknown function DUF227
CG13659	CG13659	16.59	4.89E-08	Protein of unknown function DUF227
CG16898	CG16898	11.68	2.54E-06	Protein of unknown function DUF227
CG11893	CG11893	2.27	1.37E-05	Protein of unknown function DUF227
CG31436	CG31436	2.59	3.69E-04	Protein of unknown function DUF227
CG31974	CG31974	2.85	1.03E-06	Protein of unknown function DUF227

CG10513	CG10513	2.49	1.18E-04	Protein of unknown function DUF227
CHkov2	CHKov2	2.30	5.18E-04	Protein of unknown function DUF227
CG31104	CG31104	36.74	6.61E-08	Protein of unknown function DUF227
CG3008	CG3008	2.20	2.46E-06	protein kinase
trbl	Tribbles	2.06	1.61E-03	protein kinase
CG8795	CG8795	2.08	4.44E-03	Rhodopsin-like GPCR superfamily
CG11425	CG11425	-4.3	3.81E-04	PA-phosphatase related phosphoesterase
CG3071	CG3071	-2.6	7.75E-04	G-protein beta WD-40 repeat, vesicle mediated transport, rRNA processing
CG32409	CG32409	-2.51	6.36E-04	Ribosomal biogenesis regulatory protein
B52	Serine/arginine rich protein 55	-2.02	4.22E-04	RNA-binding region RNP-1

FC:fold change. Function derived from gene ontology biological process and/or InterPro protein domain.

Table 3.14 Top 10 upregulated genes unique to caffeine

Gene Symbol	**Gene Title**	**FC**	***P* -value**	**Function**
CG31104	CG31104	36.74	6.61E-08	Protein of unknown function DUF227
GstD5	Glutathione S transferase D5	21.31	1.02E-08	Glutathione transferase
CG13659	CG13659	16.59	4.89E-08	Protein of unknown function DUF227
Hsp22/Hsp 67Bb	heat shock protein hsp22	13.41	2.76E-08	Response to stress
CG16898	CG16898	11.68	2.54E-06	Protein of unknown function DUF227
GstD6	Glutathione S transferase D6	6.34	3.01E-05	Glutathione transferase
CG6830	CG6830	5.84	5.83E-07	Protein of unknown function DUF227
CG17751	CG17751	5.36	1.74E-05	Secondary active organic cation transmembrane transporter
Cyp9b2	fly plexin a	5.07	5.84E-08	Cytochrome P450
Cyp28a5	Cyp28a5	5.03	3.89E-06	Cytochrome P450

FC:fold change. Function derived from gene ontology biological process and/or InterPro protein domain.

Table 3.15 Top 10 downregulated genes unique to caffeine

Gene Symbol	**Gene Title**	**FC**	***P* -value**	**Function**
Lsp1alpha	Larval serum protein 1 alpha	-4.42	7.71E-04	Oxygen transporter
CG11425	CG11425	-4.30	3.81E-04	PA-phosphatase related phosphoesterase
spok	Spookier	-4.24	1.55E-03	Ecdysone biosynthetic process
CG1774	CG1774	-3.51	7.74E-05	*unknown*

Cpr51A	CG10112	-2.94	6.72E-04	Insect cuticle protein
CAH2	Carbonic anhydrase 2	-2.85	1.79E-05	Carbonate dehydratase
CG8083	CG8083	-2.78	2.31E-04	Na+ dependent nucleoside transporter
CG3071	CG3071	-2.64	7.75E-04	rRNA processing
Nnp-1	Nnp-1	-2.59	8.32E-04	rRNA processing
CG12699	CG12699	-2.55	1.16E-03	*unknown*

FC:fold change. Function derived from gene ontology biological process and/or InterPro protein domain.

Table 3.16 Highly enriched gene families upregulated unique to caffeine

Gene family based on InterPro domains or GO terms	**Total gene number in genome**	**Gene number found in array**	***P* -value**
CYP	92	7	6.92897E-29
Redox	647	12	1.08781E-09
GST	45	2	3.93054E-10
UGT	38	3	6.99953E-15
Secondary active organic cation transmembrane transporter	24	4	1.26963E-37
ABC transporter	23	1	0.002271939
DUF227	41	11	4.0465E-142
Mo-molybdopterin cofactor biosynthetic process	7	2	4.4369E-33
Long chain fatty acid transporter	8	1	3.70025E-08

Table 3.17 Highly enriched gene families in 59 genes downregulated unique to caffeine

Gene family based on InterPro domains or GO terms	**Total gene number in genome**	**Gene number found in array**	***P* -value**
RNP	156	3	3.13E-04
rRNA	42	3	2.49E-15
Ribosomal assembly	40	1	9.38E-03
G-protein beta WD-40 repeat	162	3	4.46E-04
ABC transporter	23	1	5.45E-04

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Chapter 4

Conclusion and Significance

My work has led to the identification of components of SMC5/6 as critical players conveying resistance to genotoxic stress in *Drosophila*. The key questions I addressed in my PhD work were: Is the caffeine sensitivity observed in caffeine-sensitive mutants (SMC5/6 and others) due to defects in DNA damage checkpoint pathways? If so, which DNA damage checkpoint pathway(s) and/or repair mechanism(s) are affected in these mutant animals? Do these caffeine-sensitive mutants have defects in caffeine detoxification? What pathway(s) is/are involved in the transcriptional induction of caffeine-responsive genes in *Drosophila*?

We were the first group to isolate mutants of *Smc5*, *Smc6* and *MAGE* in *Drosophila melanogaster* and showed that the Smc5/6 complex is not essential for viability but plays a conserved role in protecting against genotoxic agents. Smc5/6 is not required for cell cycle checkpoint response. Rather it is involved in the homologous recombination repair pathway (*1*). Mutants of the Smc5/6 complex did not seem to have defects in caffeine-induced detoxification response, tested at a time prior to uncovering the true identities of these mutations. Unrepaired DNA damage results in genome instability and can lead to the formation of cancer. Thus it is of critical importance to gain insights into cellular surveillance mechanisms such as cell cycle checkpoints and the molecular basis of DNA repair (*2*). Studies of protein complexes that are critical for cellular responses to genotoxic stress are also highly relevant to cancer therapy in humans. It is increasingly apparent that the gene expression signature of each tumor dictates in part the success or failure of chemotherapeutic treatment or radiotherapy (*3*). The expression of human Type I MAGE genes is commonly dysregulated in cancer

cells. Moreover, many studies have correlated the levels of expression of particular MAGE genes with therapeutic response, prognosis and probability of metastasis (*4*). The unexpected link between loss-of-SMC5/6 function and caffeine could potentially be exploited for new therapeutic approaches where one could possibly sensitize checkpoint-compromised cancer cells to apoptosis, thus promoting them to "self-destruct".

My other project was aimed at establishing caffeine as a tool to comprehensively study detoxification responses in *Drosophila*. When I started the project, no genome-wide studies using caffeine had been published, but this changed when other labs (*5*) published a series of experiments detailing the effects of caffeine in larvae and cell culture. However, my original goal was to identify a gene set of caffeine-responsive genes that could be used (by my successors) to identify primary and secondary response genes, and define caffeine-specific *cis*-regulatory elements within those primary genes using an *in silico* approach based on MEME (*6, 7*) or similar programs. The holy grail was to identify transcription factors that regulate xenobiotic detoxification in *Drosophila melanogaster*. To this effect, another study appeared during my thesis that showed that *dNrf2/Keap1* is a key regulator of detoxification responses in *Drosophila* (*8*). My own analysis did identify this factor as well, but also suggests that additional transcriptional regulators likely contribute to the induction of xenobiotic enzymes as well.

It would be beyond the scope of my PhD thesis to characterize novel transcription factors that regulate detoxification processes in response to caffeine

or other drugs. The next step towards this goal would be to identify and confirm predicted caffeine response elements *in vivo*. However, the successful identification of such a factor and its binding sites would lay the foundation for future experiments aimed at a better understanding of how metabolic insecticide resistance is regulated in arthropods, thus ultimately paving the way for more effective pest control strategies.

Studying xenobiotic detoxification pathways could in principle aid our ability to manage insect pests in the future. Furthermore, characterizing detoxification pathways in *Drosophila* may facilitate our understanding of xenobiotic pathways in humans. This has important pharmaceutical implications, since most prescription drugs induce detoxification enzymes, and administration of one drug might induce detoxification enzymes that also degrade another drug (*9, 10*). Unraveling the regulatory hierarchy of xenobiotic pathways is therefore crucial to predict and analyze these drug-drug interactions.

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