Communication

NAP Family CG5017 Chaperone Pleiotropically Regulates Human AHR Target Genes Expression in Drosophila Testis

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Received: 31 October 2018; Accepted: 13 December 2018; Published: 29 December 2018

Abstract: To study the regulatory mechanism of the Aryl hydrocarbon receptor (AHR), target genes of transcription are necessary for understanding the normal developmental and pathological processes. Here, we examined the effects of human AHR ligands on male fecundity. To induce ectopic human AhR gene expression, we used Drosophila melanogaster transformed with human AhR under the control of a yeast UAS promoter element capable of activation in the two-component UAS-GAL4 system. We found that exogenous AHR ligands decrease the number of Drosophila gonadal Tj-positive cells. We also found both an increase and decrease of AHR target gene expression, including in genes that control homeostasis and testis development. This suggests that gonadal AHR activation may affect the expression of gene networks that control sperm production and could be critical for fertility not just in Drosophila but also in humans. Finally, we found that the activation of the expression for some AHR target genes depends on the expression of testis-specific chaperone CG5017 in gonadal cells. Since CG5017 belongs to the nucleosome assembly protein (NAP) family and may participate in epigenetic regulation, we propose that this nucleotropic chaperone is essential to provide the human AHR with access to only the defined set of its target genes during spermatogenesis.

Keywords: nucleosome assembly protein; Aryl hydrocarbon receptor; xenobiotic; spermatogenesis; CG5017; Drosophila

1. Introduction

There is evidence that the Aryl hydrocarbon receptor (AHR) plays an important role in normal development and cancerogenesis [1–14]. A proper concentration of activated AHR is important for cell survival and organism functioning [15–19]. Ligand binding is critical for AHR activation, because after the binding it moves to the nucleus, dimerizes with the Aryl hydrocarbon receptor nuclear translocator (ARNT), and starts functioning as a transcriptional factor which binds to specific DNA sequences known as the xenobiotic response elements (XRE), driving expression of its target genes [20,21]. An increased risk of cancer and the inability to protect cells against the toxic effects of xenobiotics are the most dramatic consequences of the decreased AHR expression [22,23]. Activation of AHR in inappropriate tissues and organs causes abnormal development, including disorders in the immune, nervous, endocrine, cardiovascular, and generative systems. Among AHR target genes, there are many genes that encode proteins responsible for homeostasis, and maintaining these is necessary for successful adaptation in new ecological niches [21,24]. Human and mammal AHRs are activated by different endogenous and exogenous ligands (xenobiotics), while in invertebrates, AHR is
activated only by endogenous ligands [3,24–29]. There is a wide range of affinities of xenobiotic ligands to AHR [30]. It is believed that the ligand binding affinities can modulate AHR’s ability to trigger the expression of its target genes [31]. The growth of the chemical and pharmaceutical industries has created conditions under which every person has a risk of exposure to xenobiotics. This may result in a variety of activations of ectopic AHR target genes in different tissues and at different stages of development.

Cell culture experiments do not provide a full understanding of the effects induced by AHR ectopic expression on the development of living organisms. For a better understanding of the function of human AHR in vivo, we created “humanized” Drosophila transgenic flies, which carry transgenes with the controlled expression of the human AhR gene guided by the yeast upstream activation sequence (UAS) [32]. This transgenic construct permits the induction of human AHR expression in certain Drosophila organs and cells with the help of tissue-specific GAL4-driver lines expressing a yeast GAL4 activator capable of recognizing UAS sequences and driving the transcription of downstream genes [33]. As most human AHR exogenous ligands are not capable of activating the Drosophila AHR homolog, this allows us to estimate the specificity of their action when adding them into the Drosophila food medium. It was shown that mouse AHR and Drosophila ARNT homolog (Tango) could form a functional transcriptional complex capable of inducing the dioxin-mediated activation of AHR target gene expressions in Drosophila [28].

We decided to use Drosophila as a verified model system to investigate the in vivo effects of human AHR ligands (xenobiotics) during development. In previous experiments using UAS-AhR/GAL4-driver flies, we have demonstrated that AHR activation could both increase and decrease transcription of AHR target genes in different tissues, and found that this effect depends on the developmental stage of the animal [32]. It is important to note that the effect of xenobiotics on the levels of AHR target gene activity was more clearly manifested in organs with a high number of proliferating cells. In adult organs, in which cell proliferation is complete, the actions of ligands on AHR target gene transcription levels were not detected. We found that the ligand’s effect on AHR target gene expression is mediated by the polycomb group (PcG) epigenetic chromatin regulators [32]. A similar xenobiotic effect may be expected in humans. In other words, we may expect the absence of the xenobiotic’s action on differentiated cells in humans. However, in adult humans, like in Drosophila imagoes, there are organs in which cells are continuously dividing. Some of these organs are testes. It is expected that ectopic AHR expression induced by xenobiotics may be the cause of various disturbances of spermatogenesis in humans.

In this paper, we apply Drosophila transformed with human AhR to estimate a possible negative effect induced by xenobiotics on human spermatogenesis. We demonstrated that the ectopic activation of human AHR in Drosophila testis cells caused a decrease in male fecundity, a decrease in the number of testis Tj-positive cells, and a change in the level of AHR target gene transcriptions. We concluded that exposure to AHR ligands could potentially lead to the risk of male infertility. Notably, we also found that the activation in the expression of some AHR target genes depends on the expression of testis-specific chaperone CG5017 in gonadal cells. Since CG5017 belongs to the nucleosome assembly protein (NAP) family [34] and may participate in epigenetic regulation, we proposed that this nucleotropic chaperone is essential to provide human AHR access to a defined set (but not all) of its target genes in soma during spermatogenesis.

2. Results and Discussion

2.1. The Effect of AHR Exogenous Ligands on Male Fecundity

We have previously demonstrated that Drosophila may serve as a valid model organism to investigate the complex effects of xenobiotics on human AHR functioning in vivo [32]. In order to investigate the effects of xenobiotics on male fecundity, we used Drosophila males carrying inducible human AhR (the UAS-AhR construct is described in Reference [32]) and Tj-GAL4 drivers. In Drosophila
testes, the $Tj\text{-}GAL4$ driver activates $UAS$-constructs in cells which are in connection with generative cells. We refused to use the $Nos\text{-}GAL4$ driver, which activates the $UAS$-constructs in germ-line cells since the fertility of $UAS\text{-}AhR/Nos\text{-}GAL4$ males was low even without exposure to exogenous ligands (about 50% according to our unpublished data, $n = 46$). This indicates the presence of endogenous AHR ligands capable of activating human AHR in $Drosophila$ Nos-positive cells that could potentially falsify experimental results. The fertility of $UAS\text{-}AhR/Tj\text{-}GAL4$ males raised on standard nutrient medium or fed with xenobiotic was not disturbed (100%, $n = 50$). It allowed us to study the effect of xenobiotic-mediated AHR activation on male fecundity using $Tj\text{-}GAL4$ driver.

The fecundity of $UAS\text{-}AhR/Tj\text{-}GAL4$ males fed with xenobiotics and of control $UAS\text{-}AhR/Tj\text{-}GAL4$ males (without exposure to xenobiotic) was measured by mating them to wild-type $Oregon\ R$ females and counting the number of undeveloped eggs produced per female over a four-day period. The replacement of fertilized females with virgins was performed daily. The effects of exogenous ligands on $UAS\text{-}AhR/Tj\text{-}GAL4$ males resulted in an increase in the proportion of undeveloped eggs in the first two days after ligand action (Figure 1). Most of the effect is caused by indirubin and beta-Naphthoflavone in the first two days after ligand exposure. Remarkably, these effects are completely reversible; when flies fed with the xenobiotic are shifted back to a standard diet, male fecundity is rapidly restored. The mechanism by which it is restored is not clear yet.

![Figure 1](image)

**Figure 1.** Daily effect of exogenous ligands on the proportion of undeveloped eggs from wild-type female after crossing with $UAS\text{-}AhR/Tj\text{-}GAL4$ male without exposure to ligands (control, orange), exposed to indirubin (blue), beta-Naphthoflavone (azure), indinol (green). Data correspond to the mean ± SD of three independent experiments. Asterisks mean the significant difference compared to the control group. Statistical analysis was performed using Student’s $t$-test (* $p \leq 0.05$).

Most likely, the decline in male fecundity was caused by the disturbances in testes cells involved in the formation of the functional spermatozoa. In the $Drosophila$ testis, germ-line stem cells and progenitor somatic stem cells reside at the tip of the testis, known as the apical hub [35]. Tj positive cells are important for the differentiation of the germ-line [35]. As we used a $Tj\text{-}GAL4$ driver to generate AHR misexpression, we proposed that the reason for the decrease in fecundity of $Tj\text{-}GAL4/UAS\text{-}AhR$ males in response to exogenous ligands might be due to the disruptions in division of Tj-positive cells.

To test our hypothesis, we estimated the number of Tj-positive cells in testes of $UAS\text{-}AhR/Tj\text{-}GAL4$ flies fed with AHR exogenous ligands for two days, also using control $UAS\text{-}AhR/Tj\text{-}GAL4$ flies developed on a standard medium. We found that the testes of flies fed with xenobiotics were thinner (Figure 2), and a decrease in the average number of Tj-positive cells per testis was observed in flies fed with xenobiotics for 3 days (indirubin $75.8 \pm 6.19$; $n = 18$, beta-Naphthoflavone: $86 \pm 9.7$; $n = 13$, $p \leq 0.05$).
indinol: 79.8 ± 8.1; n = 14) when compared to testes from males raised on the standard medium (106.3 ± 8.25; n = 24) (Figure 2E). No remarkable differences between testis of Tj-GAL4/+ flies fed with xenobiotic and testis of flies with the same genotype developed on standard medium were detected (Appendix A Figure A1). Thinner testes were typical for only UAS-AhR/Tj-GAL4 flies fed with xenobiotic so we attributed this effect to the ectopic AHR activation.

In Drosophila testis, the absence of Tj-positive cells blocks normal spermatogenesis [36]. Thus, the decrease in the number of Tj-positive cells in response to human AHR activation by exogenous ligands in testes of UAS-AhR/Tj-GAL4 flies could be the reason of a reduced production of spermatozoa and decreased male fecundity.
We believe that the cause of the detected functional and morphological differences between the control and experimental males should be due to the activities of the AHR targeted genes that regulate homeostasis and cell division.

2.2. The Effects of Exogenous Ligands and Testis-Specific Chaperone CG5017 on the Expression of AHR Target Genes in Drosophila Testes

To assess the ability of xenobiotics to influence the expression of human AHR target genes in Drosophila testes, we first identified potential human AHR target genes in Drosophila (described in Reference [32]). We selected several putative Drosophila homologs of human AHR targets genes containing XRE-elements in their regulatory regions: Mannosyl (α-1,3-)glycoprotein β-1,2-N-acetylglucosaminyltransferase 1 (Mgat1), which participates in the determination of adult lifespan relating to mushroom body development; Glutathione S transferase T4 (GstT4), which is involved in oxidation-reduction processes and catalyzes reactions of biotransformation; Cytochrome P450 6g1 (Cyp6g1), which is involved in the oxidation-reduction process, response to DDT, and the insecticide catabolic process; N-acetylneuraminic acid synthase (Nans), which participates in the carbohydrate biosynthetic process; Relish (Rel), which encodes the NF-κB subunit; p53, which is a transcriptional factor required for adaptive responses to genotoxic stresses, including cell death, compensatory proliferation and DNA repair; Myc, a transcription factor related to proto-oncogenes, which contributes to cell growth, cell competition, and regenerative proliferation; dacapo (dap), which encodes the Cyclin-dependent kinase inhibitor; the Retinoblastoma-family protein (Rbf), which provides negative regulation of the G1/S transition of mitotic cell cycles; Jun-related antigen (Jra), which is involved in positive regulation of the metabolic process, humoral immune response, aging, and RNA polymerase II transcription factor activity; and Dc42 (Cdc42), which is a key regulator of the actin cytoskeleton, playing a central role in actin cytoskeleton organization, morphogenesis, hemocyte migration, cell polarity, and wound repair.

To investigate the effects of exogenous ligands in vivo, we analyzed the expression of AHR target genes by RT-PCR in testes of UAS-AhR/Tj-GAL4 flies fed with xenobiotics for two days. To activate human AHR, we used exogenous ligands known to act as agonists of this receptor. This means that these molecules only cause an increase in the transcription levels of AHR target genes [20,37]. We found that induced human AHR had pleiotropic effects on its target genes, depending on the nature of the exogenous ligand. In other words, the xenobiotic-mediated effect of human AHR activity in testes of UAS-AhR/Tj-GAL4 flies resulted in three different ways: Some experienced a decrease in the gene expression, some an increase in gene expression, and several genes had no response to AHR activity (Figure 3). For example, the activation of human AHR by indirubin resulted in the activation of almost all genes tested except Mgat1, Cyp6g1, and Myc. The activation of human AHR by beta-Naphthoflavone resulted in the activation of Cyp6g, Rel, and Myc, and the suppression of Mgat1 and dap genes. The activation of the human AHR by indinol resulted in the suppression of Cyp6g and the weak activation of Mgat1, GstT4, Csas, Rel, p53, Myc, and Jra genes.
To test this, we performed experiments using UAS-AhR/Tj-GAL4
CG5017 previously shown that NAP family
ligands for 2 days. Flies of the same genotype developed on the standard nutrient medium were
mRNA levels of AHR target genes were measured by RT-PCR in testes of
flies we added indirubin, beta-Naphthoflavone, and indinol into the nutrient medium. The
synergy in the genetic interactions between hypomorphic mutations of
NAP family nucleotropic chaperones which control the activity of H2A-H2B histones [34,37–40]. It was
mediated by the polycomb group (PcG) epigenetic chromatin regulators [32].

In our previous study, a similar effect was found [32]. We attributed this effect to the epigenetic
repressive state of chromatin, which limits the ability of a human AHR to access XREs and control its
target gene expression in the Drosophila genome. This hypothesis was confirmed in our experiments,
through which we demonstrated that the effects of exogenous ligands on AHR target genes are
mediated by the polycomb group (PcG) epigenetic chromatin regulators [32].

The formation of the epigenetic state of genes involved not only Pc and Trx complexes, but also
NAP family nucleotropic chaperones which control the activity of H2A-H2B histones [34,37–40]. It was
previously shown that NAP family CG5017 and spineless (ss, D. melanogaster homologue of mammalian
AhR) act synergistically, controlling morphogenesis, memory, and detoxification [41,42]. The synergy in
the genetic interactions between hypomorphic mutations of ss and CG5017 may reflect the involvement
of NAP family chaperones in the regulation of AHR-signaling in D. melanogaster. We decided to study
the effect of CG5017 on human AHR target gene transcription in somatic cells of Drosophila testis.

To test this, we performed experiments using UAS-AhR/Tj-GAL4 flies carrying a mutant hypomorphic
allele of CG5017-ssaSc [43,44]. To activate human AHR in UAS-AhR/Tj-GAL4; ssaaSc flies we added
indirubin, beta-Naphthoflavone, and indinol into the nutrient medium. The mRNA levels of AHR
target genes were measured by RT-PCR in testes of UAS-AhR/Tj-GAL4; ssaSc flies fed with exogenous
ligands for 2 days. Flies of the same genotype developed on the standard nutrient medium were
used as a control. A remarkable increase in the transcription of some AHR target genes was observed
(Figure 4).
The activation of human AHR by beta-Naphthoflavone de-repressed very pronounced and resulted in de-repression of Cyp6g1.

On the other hand, the transcription levels of Cyp6g1 (up to 22 times), and omitted the silencing of GstT4, Csas and Nans. The activation of human AHR by indinol is not very pronounced and resulted in de-repression of Cyp6g1. On the other hand, the transcription levels of genes regulating cell proliferation and differentiation were either not affected or were decreased (Figure 4, Table 1).

**Table 1.** The decreased expression of nucleotropic chaperone CG5017 activates ligand-dependent transcription of some AHR target genes. Summarized results of real-time PCR experiments shown on Figures 3 and 4. «+» and «0» columns represent results shown on Figures 3 and 4 respectively. «+», «−» and «0» mean the increasing expression, the decreasing expression and no effect, respectively. Red pluses mean the remarkable increasing in transcription on the background of mutant CG5017.

| Gene Symbol | Indirubin | beta-Naphthoflavone | Indinol |
|-------------|-----------|---------------------|---------|
| Allele of CG5017 | +/+ | ss<sup>aSc</sup> | +/+ | ss<sup>aSc</sup> | +/+ | ss<sup>aSc</sup> |
| Mgat1       | 0         | −                   | −       | +       | +       | +       |
| GstT4       | +         | 0                   | 0       | +       | +       | +       |
| Cyp6g1      | 0         | +                   | +       | −       | +       |
| Csas        | +         | 0                   | 0       | +       | 0       |
| Nans        | +         | 0                   | 0       | +       | 0       |
| Rel         | +         | 0                   | +       | +       | +       | 0       |
| p53         | +         | 0                   | 0       | −       | +       | −       |
| Myc         | 0         | +                   | +       | −       | +       | −       |
| dap         | +         | 0                   | 0       | −       | −       | 0       |
| Rbf         | +         | 0                   | 0       | −       | 0       | −       |
| Jra         | +         | 0                   | 0       | +       | −       |
| Cdc42       | +         | +                   | 0       | −       | 0       | −       |

**Figure 4.** Decreased expression of CG5017 nucleotropic chaperone leads to ligand dependent activation in transcription of some AHR target genes. Relative mRNA levels were analyzed by real-time PCR in testes dissected from UAS-Ahr/Tj-GAL4; ss<sup>aSc</sup> flies fed with indirubin, beta-Naphthoflavone or indinol for 2 days. UAS-Ahr/Tj-GAL4; ss<sup>aSc</sup> flies developed on standard medium were used as a control. Transcript levels are represented as means ± SD (error bars). *p < 0.05, compared to the control. Statistical analysis was performed using Student’s *t*-test.

Activation of AHR by xenobiotics on the background of a mutant allele of CG5017 omitted the silencing of AHR target genes involved in maintaining cell homeostasis (Table 1). For example, the activation of human AHR by indirubin resulted in strong activation of Cyp6g1 (up to 22 times). The activation of human AHR by beta-Naphthoflavone de-repressed Mgat1 (up to 3–5 times), and omitted the silencing of GstT4, Csas and Nans. The activation of human AHR by indinol is not very pronounced and resulted in de-repression of Cyp6g1. On the other hand, the transcription levels of genes regulating cell proliferation and differentiation were either not affected or were decreased (Figure 4, Table 1).
The regulatory mechanism of AHR target gene expression by CG5017 is not clear. Since CG5017 belongs to the nucleosome assembly protein (NAP) family [34] and may participate in epigenetic regulation, we proposed that this nucleotropic chaperone could be essential to enable human AHR to access only a defined set of its target genes in soma during spermatogenesis.

Our results indicate a complex, multi-step regulatory mechanism of proper AHR target gene transcription which can be disrupted by exogenous ligands, which may be the cause of many diseases [45]. We hope that further study of the exogenous ligands’ action mechanisms will help in the development of strategies for limiting xenobiotic effects and reducing pathology.

3. Materials and Methods

3.1. Fly Stocks, Rearing Conditions, Reagents and Crosses

UAS-AhR strain with inducible human AhR gene expression in D. melanogaster genome was generated early [32]. Wild type Oregon R and Tj-GAL4/Cy strains were obtained from Bloomington Drosophila stock center. Also, we used ss^Sc strain with hypomorphic mutations of CG5017 and spineless genes [43,44].

Flies were reared on nutrient Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC, USA). Following ligands were used: 2′Z-Indirubin (Sigma-Aldrich, St. Louis, MO, USA), beta-Naphthoflavone (Thermo Fisher Scientific, Waltham, MA, USA), Indole-3-Carbinol (Mirax Biopharma, Moscow, Russia). Ligand solutions were prepared as described in [32]. Final concentrations of beta-Naphthoflavone, indirubin and indole-3-carbinol were 200 µg/g medium, 25 µg/g medium, 10 µg/g medium correspondently.

Ligands were fed to imago F1 offspring after the crossing of Tj-GAL4 males with UAS-AhR females. Parents were kept on standard Formula 4-24 medium. After hatching flies of first day old were selected for feeding experiments. Flies were kept at room temperature (25 °C).

To obtain flies of UAS-AhR/Tj-GAL4; ss^Sc genotype we crossed UAS-AhR/Cy; ss^Sc/D females with Tj-GAL4/Cy; ss^Sc/D males and flies without balancer chromosomes were further selected in the F1 offspring. Flies were kept at room temperature (22 °C).

3.2. Calculation of Undeveloped Eggs Frequency

Reproductive output was measured at 25 °C. Imago males Tj-GAL4/UAS-AhR (n = 7) were fed with ligands solutions for 2 days whereupon males were crossed with Oregon R virgin females (n = 7) in fresh medium vials. During four days we replaced fertilized Oregon R females with virgin ones after 24 hr and counted the total number of eggs laid. We considered unfertilized eggs that did not develop within 24-25 hr. The experiment was performed three times. The proportion of daily undeveloped eggs per female was calculated using the following formula: [(Number of undeveloped eggs/Total number of eggs) × 100%]/[Number of females tested].

3.3. Real-Time Reverse-Transcription PCR Analysis

Experiments have been done in triplicate as described previously [32]. Primers and TaqMan® probes used for RT-qPCR experiments are available in Appendix A Table A1.

3.4. Immunohistochemistry

Experiments have been done as described previously [32]. Primary antibody used in our work is guinea-pig polyclonal anti-Tj (1:5000) [46]. Secondary antibodies (1:200) were conjugated to Alexa Fluor–488 (Molecular Probes, Waltham, MA, USA). DNA was stained with SytoxGreen (1:500, Thermo Fisher Scientific, Waltham, MA, USA).
3.5. Microscopic Analysis

The resulting immunostaining preparations were examined using Leica TCS SP5 confocal microscope using a multichannel mode with a 40 × immersion oil lens. The images were recorded with a z-resolution of 0.7–0.8 μm.

3.6. Image Analysis

The resulting images were imported into Imaris® 5.0.1 (Bitplane AG, Belfast, UK) for further processing. Estimation of somatic cells on the confocal images was carried out by measuring number of Tj-stained cells. Student’s t-tests were used for confirmation of statistical significance. The threshold of statistical significance was $p \leq 0.01$ for beta-Naphthoflavone and $p \leq 0.001$ for indirubin and indinol.

Author Contributions: Conceptualization, O.B.S. and B.A.K.; Data curation, J.E.V., R.O.C., O.B.S. and B.A.K.; Formal analysis, A.A.A., J.E.V., R.O.C. and M.S.S.; Funding acquisition, J.E.V. and O.B.S.; Investigation, A.A.A., J.E.V., R.O.C. and M.S.S.; Methodology, A.A.A., J.E.V., R.O.C. and M.S.S.; Project administration, O.B.S. and B.A.K.; Resources, J.E.V., R.O.C., M.S.S., O.B.S. and B.A.K.; Software, A.A.A., J.E.V. and R.O.C.; Supervision, B.A.K.; Validation, A.A.A., J.E.V. and R.O.C.; Visualization, A.A.A. and J.E.V.; Writing—original draft, A.A.A., J.E.V., R.O.C., O.B.S. and B.A.K.; Writing—review & editing, O.B.S. and B.A.K.

Funding: This research was funded by RFBR (project No. 16-04-00829-a, 18-34-00162 mol-a) and was partly conducted in the frame of IDB RAS government program of basic research No. 0108-2019-0001.

Acknowledgments: The authors thank Bloomington Drosophila stock center for the providing Drosophila stocks. We are grateful to Dr. Dorothea Godt for the providing of anti-TJ antibodies. We are grateful to Dr. Lyudmila Olenina for the help in calculation of Tj-positive cells.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AHR Aryl Hydrocarbon Receptor
NAP Nucleosome Assembly Protein
TJ Traffic jam
UAS Upstream Activating Sequence

Appendix A

Table A1. Sequences of primer pairs and TaqMan® probes used in this study. FAM fluorescent dye (6-carboxy fluorescein) was used for labeling oligonucleotides. BHQ1 (Black Hole Quencher-1) is used as a dark quencher.

| Gene symbol | Title | Sequences (5′–3′) |
|-------------|-------|------------------|
| Csas        |       |                  |
| CsasT1      | GAAATTGGTGCTCATCGCT |
| CsasT2      | GGAACTCCGAAATGGCATGA |
| CsASTAQ     | FAM-CGTCTCTGGCGAATTTCTCAGGCCG-BHQ1 |
| Nans        |       |                  |
| NanSt1      | TGGCCTGCGAAATAAACTG |
| NanSt2      | TCCAAGAAATCCTCGGTG |
| NanSTAQ     | FAM-AGTCAGTGAAACCGCGGCCT-BHQ1 |
| MgtAT1      |       |                  |
| mgat1f      | TGAATTTCAAGACCGTGTTTC |
| mgat1r      | GGCCTACTCTGTCCCTAGC |
| mgataq      | FAM-TACAACAAAAACCGCGCGTCA-BHQ1 |
| GstT4       |       |                  |
| cg1681f     | TTCGCACCCACTCTAGTCAC |
| cg1681r     | GCTCGATTGGTTCAGGAAAT |
| cg1681taq   | FAM-TCAACGAGATGTCGCGCCACTC-BHQ1 |
Table A1. Cont.

| Gene symbol | Title | Sequences (5′–3′) |
|-------------|-------|------------------|
| **Cyp6g1** |       |                  |
| Cyp6g1f     |       | GCGATCCATGCTAGATAGATAT |
| Cyp6g1r     |       | CCAATTCCTGCTGATAAGGTT |
| Cyp6g1taq   |       | FAM- TCGCACCAAGCTGACTCCCG-BHQ1 |
| **Rel**     |       |                  |
| Relf        |       | GAAAGTACGATGCTGTCGA |
| Relr        |       | TGTCTGTCCATTCCGTTGCTC |
| Reltaq      |       | FAM-TCGCGCCAACTCGCGTTA-BHQ1 |
| **p53**     |       |                  |
| p53f        |       | GTACTCGATTCCGCTGAACA |
| p53r        |       | CACGCAAATAAGTGGTTGG |
| p53taq      |       | FAM-CTGAACGTCCAGTTGAAGGCC-BHQ1 |
| **Myc**     |       |                  |
| dmf         |       | CCGCGCTACAATAACTCCTAA |
| dmr         |       | GCAGTTCTGATACGGTGTGC |
| dmtaq       |       | FAM-CGCGCCAACTCGCGTTA-BHQ1 |
| **dap**     |       |                  |
| dacf        |       | CAGAGATGTACACCCTAA |
| dacr        |       | GGAGTCGTAACAAGATTC |
| dactaq      |       | FAM-TTATCCGTGTTCGACTCTAGCG-BHQ1 |
| **Jra**     |       |                  |
| jraf        |       | TTCACACTAACTCCAGGCA |
| jrar        |       | CTGGTCATGTTGTAGG |
| jrataq      |       | FAM-CAACTCGCCAGCCAGCA-BHQ1 |
| **Rbf**     |       |                  |
| Rbf         |       | CTGGCGGAAGAGATAGCC |
| Rbr         |       | GGACTTCGCTAGTTGGAAGC |
| Rbtaq       |       | FAM-CGCGCCAACTCGCGTTA-BHQ1 |
| **Cdc42**   |       |                  |
| cdc42f      |       | CGAGATTACACACCAGGCC |
| cdc42r      |       | ATGGGCTTCTGCTGTTGTC |
| cdc42taq    |       | FAM-TTATCCGTGTTCGACTCTAGCG-BHQ1 |
| **Rpl32**   |       |                  |
| Rpl32dir    |       | CCAGCATAAGCCGCAAGATC |
| Rpl32rev    |       | ACGCACCTCTGTGGTCAGATC |
| Rpl32probe  |       | FAM-CGCGCCAAAGCCACTCCGCGCAC-BHQ1 |

**Figure A1.** Merged confocal immunofluorescence images of apical tips of testis stained with SytoxGreen to highlight DNA (green) and anti-Tj to visualize Tj-positive cells (red). Testes are from *Tj-Gal4/+* males: (left) raised on standard medium; (right) fed for 3 days with indirubin. A number of Tj-positive cells in testis of flies developed on standard medium or fed with xenobiotic was the same (100.1 ± 5.2; *n* = 22 and 104.8 ± 7.7; *n* = 19 correspondently). Scale bars, 30 µm.
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