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Review Article
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Understanding the Mechanisms Involved in the Regulation of Cytochrome P450 Gene Expression in *Drosophila melanogaster* (Diptera: Drosophilidae)

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Abstract

Cytochrome P450 monooxygenases (P450s) are known to play a central role in the adaptive response of insects and other animals to chemicals in the environment. *Drosophila* spp. P450s are known to be regulated by Cap 'n' collar isoform C (CnCC) and/ Spineless (ss) which are orthologs to Nuclear factor erythroid-2 factor 2 (Nrf2)/Aryl hydrocarbon receptor (AhR) in higher mammals. However, the mechanism underpinning this regulation in insects including fruit fly, *Drosophila melanogaster* is poorly understood. Understanding the constitutive and inducible patterns of expression requires knowledge about the signalling pathways that control insect P450 expression, which is still lacking for most identified insect P450s. *D. melanogaster*, because of its longstanding use as a genetic model insect, is a powerful tool for identifying possible regulatory mechanisms and for following expression through to function. Here, we describe the roles played by the cis-acting elements and the Transcription Factors (TFIF) mechanisms involved in the regulation of cytochrome P450 genes in *D. melanogaster* in response to xenobiotic compounds. These cis-acting elements include; promoters, enhancers, repressors, silencers and insulators. The regulatory mechanisms involved in the regulation of the P450s by the spineless (ss)/tango (Tgo) and CnCC/dKeap1 (*Drosophila* Kelch-like ECH-associated protein 1) signalling pathways in insecticide resistance were also extensively discussed. This review increases our understanding of the regulatory mechanisms involved in the insecticide metabolism in *Drosophila melanogaster*.

Keywords: CnCC; dKeap 1; *Drosophila melanogaster*; p450s; Spineless; Tango

Introduction

The cytochrome P450 superfamily consists of a considerable amount of heme-containing monooxygenases and is established in all living organisms including insects [1,2]. In the fruit fly, *Drosophila melanogaster* alone there are 83 P450 genes with a few of these enzymes playing key roles in the metabolism or activation of xenobiotics [3-6]. These P450s include; *Cyp6a2* (substrate-DDT, aldrin, dieldrin and diazinon) [7-9] Cyp6g1 (substrate-DDT, imidacloprid) [10,11]. *Cyp12a4* (substrate-Lufeneron) and *Cyp12d1* (substrate-DDT and carbaryl) [12-14]. In addition, P450s also partake in the biosynthesis of cuticular hydrocarbons, ecdysteroids, juvenile hormone, and pheromones [15,16]. In higher mammals, some of these xenobiotic metabolizing cytochrome P450 genes are established to be upregulated by the transcription factors such as the Aryl hydrocarbon receptor (AhR)/Aryl hydrocarbon receptor nuclear translocator (ARNT) and/or Nuclear factor erythroid-2 related factor-2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap 1) [17-21]. Interestingly, AhR/ARNT and Nrf2/Keap 1 display a multilevel crosstalk where the latter is also a target of the former [22-24]. Previous studies also revealed the orthologs to Aryl hydrocarbon receptor/(AhR) Aryl hydrocarbon nuclear receptor (ARNT) and Nuclear factor erythroid factor 2 (Nrf2)/Kelch-like associated protein 1(Keap 1) in *Drosophila melanogaster* to be Spineless (Ss)/Tango (Tgo) and Cap 'n' collar isoform C (CnCC)/*Drosophila* Kelch-like ECH-associated protein 1 (dKeap 1) respectively [25-28]. Therefore, to understand the mechanism by which these P450s are regulated in *Drosophila melanogaster* (an insect model) the basics underpinning this regulation are described below:

Regulation of Gene Expression

The fundamental component of the control of gene expression is attained at the transcriptional level [29]. This level of regulation consolidates the contribution of various types of cis-acting genomic elements, which are vital molecular switches involved in the transcriptional regulation of a productive chain of gene activities regulating numerous biological processes, including abiotic stress responses, developmental processes and hormone responses [30,31]. This transcriptional regulation transpires within a complex genomic milieu in which promoters, enhancers, and insulators are intimately connected both along the one-dimensional linear chromosome and within the three-dimensional nuclear chromatin environment [32-34].

Promoter

Regulation of gene expression at the promoter level is chiefly regulated by the cis-acting elements restricted upstream of the transcriptional start site [35]. The physical interplay between regulatory proteins and the basic transcriptional machinery is straight forward during initiation of transcription owing to the location of proximal elements to the core promoter [34].

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Enhancers

In variance with promoters, enhancers are typically located far away from the genes they regulate [31,32]. Albeit a promoter is utterly vital for gene transcription, a significant part of metazoan transcriptional regulation emanates via the action of distal cis-regulatory modules [33,36].

Repressors

Repressors appear to function by blocking the binding of a nearby activator, or by directly competing for the same site [36,37]. It has been proposed that the difference between the two may be associated to the recruitment of distinctive cofactors [36].

Silencers

Silencers are binding sites for negative transcription factors termed repressors [36]. Silencers are sequence-specific elements that confer a negative (i.e., silencing or repressing) effect on the transcription of a target gene [38]. Repressor function can require the recruitment of negative cofactors, also termed co-repressors, and in some cases, an activator can switch to a repressor by differential cofactor recruitment [36]. In *Drosophila*, two classes of silencers have been observed: short-range silencers, which generally must reside within ~100 bp of their target gene to have a repressive effect and long-range silencers, which can repress multiple enhancers or promoters over a span of a few kilo base pairs [36].

Insulators

A third critical component contributing to gene expression is the insulator. Originally defined in *Drosophila*, and still best understood in that organism, insulators were so named due to their ability to "insulate" genes from position effects in transgenic assays. Historically, two major roles have been ascribed to insulator elements: the ability to serve as boundary elements preventing the spread of heterochromatin, and the ability to prevent enhancer activity when interposed between an enhancer and promoter [33].

Regulatory mechanismS in resistance

Drosophila melanogaster has been used broadly as a model system to understand the molecular mechanisms underlying insecticide resistance [25]. Further studies also addressed the mechanisms that underlie this regulation, mapping critical promoter elements that are required for P450 gene induction in response to pesticides or the well-studied xenobiotic phenobarbital (PB) [39-45]. The Spineless/Tango and CnCC/dKeap1 signaling pathways are hereby described below.

The Spineless (Ss) gene

The *Drosophila* gene spineless (Ss) is the ortholog of vertebrate AhR [26,46]. These proteins share extensive sequence identity, especially in their Basic Helix-Loop-Helix (bHLH) regions, and must share common ancestry, as several of the splice sites in the Ss and AhR genes are precisely conserved [47].

Function and structure of spineless: Like other invertebrate homologs of AhR, spineless does not bind prototypical xenobiotic ligands of the vertebrate receptor such as 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) [48]. Studies revealed that spineless, functions as a heterodimer with Tango, the *D. melanogaster* ortholog of ARNT and both appear to recognise the same DNA sequence, the xenobiotic response element (XRE), a core nucleotide sequence at the upstream of inducible target genes for the transcription factor Aryl Hydrocarbon receptor (AhR) that is responsible for recognition of exogenous environmental pollutants in eukaryotic cells [49,50]. Furthermore, Tango heterodimerize with Trachealess and Single minded (both bHLH-PAS family members) and regulates transcription in the trachea and central midline, respectively [51].

Ligands of spineless (AhR): Spineless (Ss) in *D. melanogaster* does not show the ability to bind to toxic agonists such as dioxin congener (TCDD) and Polycyclic Aromatic Hydrocarbons (PAHs) [52]. It is therefore plausible that, as a consequence of their toxic activity, an endogenous ligand competent of triggering the Ss protein is generated. Such a role is often played by one of the endogenous AhR ligands–a toxic tryptophan derivative Formyl-Indolo-Carbazole (FICZ). Consequently, cellular concentrations of FICZ levels are elevated considerably in response to ionizing radiation and this inevitably triggers elevated expression of the Ahr gene thereby leading to cellular reaction to toxin exposure, and, in particular, to ionizing radiation. This induces expression of detoxification-related genes *Cyp6g1* and *Cg1681,* hence ss gene is necessary for this induction [47]. This protein can however bind to the XRE and stimulate transcription from genes containing this cis-acting element [49,53]. Moreover, it regulates normal morphogenesis of the leg or antenna and bristles, all of which are dominant *Drosophila* sensor organs or tissues that operate in response to environmental chemicals [54].

Molecular Mechanisms of AhR Functions in the Regulation of Cytochrome P450S in *Drosophila*

Spineless (Ss) Tango (Tgo) signalling pathway

The spineless (Ss) protein remains predominantly cytoplasmic as part of a protein complex with the molecular chaperone heat shock protein 90 (HSP90), p23, and XAP2. It is known to interact with Tango (Tgo), the fly homolog of mammalian ARNT and through this interaction the protein is transported to the nucleus from the cytoplasm where it binds another bHLH-PAS-protein, the Aryl hydrocarbon receptor nuclear translocator (ARNT). The Ss: Tgo heterodimer binds to a specific motif, XRE in the promoters of its target genes and regulates their transcription [47,55]. The Ss: Tgo heterodimer can both repress and activate explicit genes, demonstrating the heterodimer's interplay with other transcription or nucleosome assembly factors [47]. AhR gene is exceedingly conserved between vertebrates and invertebrates (Figure 1) [47].

The Cap 'n' Collar Isoform C (CnCC) (named after the CnC gene of Drosophila)

Studies of the *Drosophila* orthologs to Nrf2 and dKeap1 have provided insights into the functions of this protein. The *Drosophila* Cap 'n' collar locus encodes CnCC, which contains a bZIP domain homologous to that of Nrf2, N-terminal DTG (Asp-Thr-Gly), a low-affinity motif and a high-affinity ETGE (Glu-Thr-Gly-Glu) motif, separated by a central lysine-rich α-helix and are homologous to those that mediate Nrf2 interaction with Keap1 (a member of the Kelch family of actin binding proteins, named after the fruit fly's Kelch protein (a component of the egg chambers) [56].

Function of the CnCC: CnCC regulates the transcriptional responses to xenobiotic compounds whilst the CnCC-Keap1 (dKeap 1) protein complexes regulate the native cellular and developmental processes [18,57].

Ligands of CnCC: DKeap1 can function as a sensor of oxidants and electrophiles, which react with its redox sensitive cysteine residues

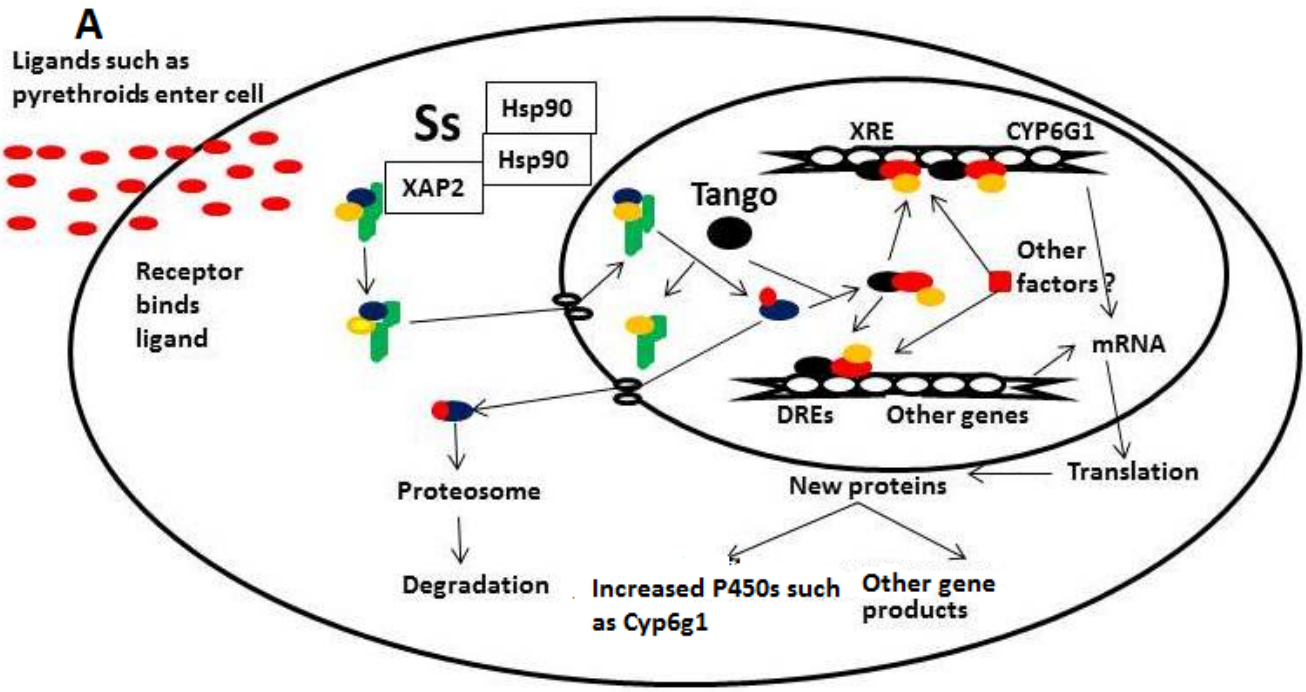

Figure 1: Schematic representation of Ss/tgo signalling pathway. The cytosolic ss are complexed of two molecules of heat shock protein (Hsp90), X associated protein (XAP2) and the co-chaperone p23 for the HSP90. Binding of a ligand, e.g. TCDD, leads to a conformational change, thereby allowing nuclear translocation of the spineless complex. In the nucleus, Spineless dissociates from the complex and dimerizes with Tango. The *Ss*–Tgo heterodimer then binds to xenobiotic response elements (XREs) in the promoters of genes encoding for several phase I and phase II metabolizing enzymes but also several other genes, e.g. *Cyp6g1* (Adopted and modified from Denison and Nagy) [64]. Schematic presentation of the sequence conservation between AhR and Ss, LBD, dioxin binding domain. (Adapted and modified from Céspedes et al. [52].

[58,59]. During oxidative stress or electrophilic xenobiotics, dKeap 1 undergoes destruction releasing CncC that translocates to the nucleus [60,61]. CncC is then stabilized and accumulates in the nucleus, where it binds to the Antioxidant Response Element (ARE) in the enhancers of its target genes [59,62].

Molecular Mechanisms of CNCC Functions in the Regulation of Cytochrome P450 genes

CnCC/dKeap1 signalling pathway

Antioxidant Response Element (ARE)-mediated response to oxidative stress is conserved from flies to humans. In unstressed conditions, Nrf2 (Nuclear factor erythroid-2 related factor- 2) in mammals, CncC (Cap 'n' collar isoform C) in *Drosophila,* is repressed by dKeap1 (*Drosophila* Kelch-like ECH-associated protein 1), which also functions as a sensor of oxidants and electrophilic compounds [59,63]. Under oxidative stress conditions, the inhibition of CncC by dkeap1 is abolished allowing this transcription factor to bind, with other proteins, to ARE sequences upregulating downstream genes such as P450s. The *Drosophila* dKeap1 contains Kelch repeats homologous to those that mediate Keap1 interaction with Nrf2 as well as a sequence motif that is required for mammalian Keap1 export from the nucleus [18]. Overexpression of CncC and depletion of dKeap1 in *Drosophila melanogaster* activates the transcription of many genes including *Cyp6g1* and *Cyp6a2* that protect cells from xenobiotic compounds, whereas dKeap1 overexpression represses their transcription, indicating that the functions of these protein families in the xenobiotic response are conserved between mammals and *Drosophila* (Figure 2A-2C) [18,25].

S/No.	Cytochrome P450	Signalling pathway	Reference
1.	CYP6G1	ss/tango and CnCC/dKeap 1	[14,47]
2.	CYP6B1	ss/tango	[66]
3.	CYP6G2	CnCC/dKeap 1	[14]
4.	CYP12D1	CnCC/dKeap 1	[14]
5.	CYP6A2	CnCC/Keap 1	[25]
6.	CYP6A8	CnCC/Keap 1	[25]
7.	CYPA21	CnCC/Keap 1	[25]
8.	CYP6BQ9	CnCC/Keap 1	[25]
9.	CYP12A4	CnCC/Keap 1	[25]

Table 1: Cytochrome P450s modulated by Spineless/Tango and CnCC/dKeap1 pathways.

Previous studies revealed that cytochrome P450 family members are modulated by Spineless/Tango and CnCC/ dKeap 1 pathways in *Drosophila melanogaster* (Table 1).

Further studies have also shown that cytochrome P450 family genes contain elements responsive to the Spineless/Tango and CnCC transcription factors in *Drosophila melanogaster* (Table 2) [64-66].

Conclusions

Here we have reviewed the role of the spineless/tango and CnCC/ dKeap 1 signalling pathways for their mechanistic role in the regulation of Cytochrome P450s in the activation of xenobiotics in *Drosophila melanogaster*. Since *D. melanogaster* is a model insect system and the

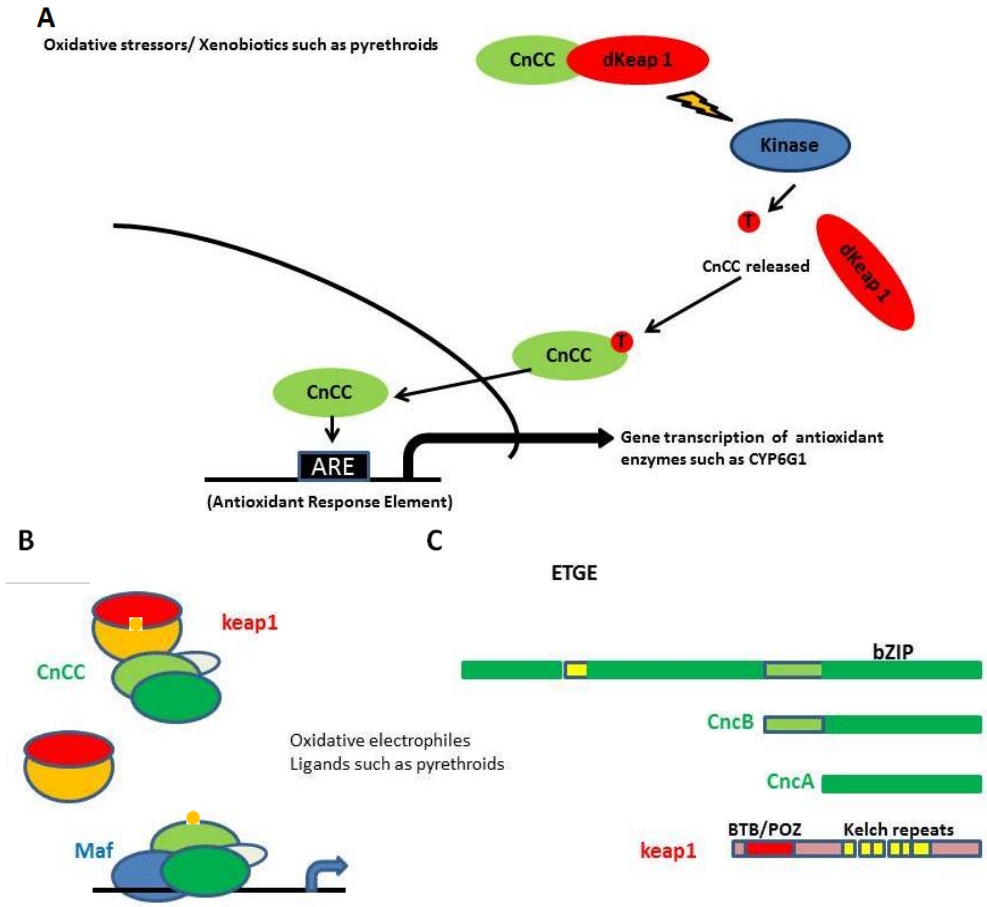

Figure 2: (A) General scheme for the induction of CnCC/dKeap 1-signaling pathway. The antioxidant response element (ARE) in the promoter region of select genes allows the coordinated upregulation of antioxidant and detoxifying enzymes in response to oxidative/electrophilic stress. This upregulation is mediated through Cap 'n' collar isoform C nuclear (CnCC) that may be activated by endogenous and exogenous molecules or stressful conditions. These agents disrupt the association between CnCC and dKeap1 with subsequent nuclear translocation of CnCC. In the cell nucleus CnCC interacts with small MAF protein, forming a heterodimer that binds to the ARE sequence in the promoter region and upregulates transcription of many genes encoding detoxifying enzymes such as *Cyp6g1*. It is therefore speculated that this signalling pathway is constitutively upregulated in long-lived individuals providing extension of longevity and health span. (Adopted and modified from [59,65]. **(B)** A detailed view at the promoter level of teh binding of CnCC to sMaf. **(C)** An illustration of the conservation of Nrf2 and Keap 1 in *Drosophila* (Adapted and Modified from [59,65].

S/No.	P450 Family genes	Responsive elements	References
1	CYP6G1	XRE	[47]
3.	CYP6G2	ARE	[25]
4.	CYP12D1	ARE	[14]
5.	CYP6A2	ARE	[25]
6.	CYP6A8	ARE	[25]
7.	CYPA21	ARE	[25]
8.	CYP12A4	ARE	[25]

XRE: Xenobiotic Response Element; ARE: Antioxidant Response Element

Table 2: Response Elements for Spineless/Tango and CnCC/dKeap 1 transcription factors.

mechanisms of insecticide resistance in this species have been studied widely, this work has implications for the mechanistic understanding of the basis of insecticide resistance in insect disease vectors and hence the spread of Vector Borne Diseases.

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