Drosophila melanogaster has the enzymatic machinery to make the melanic component of neuromelanin

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Dopamine is a vital catecholamine for neurotransmission in animals. In human brains, it plays a crucial role in regulating motor activity, the process of learning, and self-stimulation. Alterations in dopamine metabolism disturb the neuronal redox homeostasis and are linked to motor impairment and mood disorders (Montes, Rivera-Mancia, Diaz-Ruiz, Tristan-Lopez, & Rios, 2014; Xu & Chan, 2015). In mammalian substantia nigra (SN), dopamine is normally sequestered into synaptic vesicles by the transporter VMAT2. The interior of these vesicles is slightly acidic, which prevents dopamine autoxidation. Nonvesicular dopamine that is not secreted for neurotransmission is eventually oxidized to form a dark brown neuromelanin. Therefore, neuromelanin is considered to have a neuroprotective role as the accumulation of dopamine quinone and related quinonoid products would result in the degeneration of the dopaminergic neurons by causing oxidative stress, proteasome dysfunction, neuroinflammation, and formation of neurotoxic oligomers of alpha-synuclein (Blesa, Trigo-Damas, Quiroga-Varela, & Jackson-Lewis, 2015; Sulzer et al., 2000; Zecca, Zucca, Albertini, Rizzo, & Fariello, 2006; Zecca, Gallorini et al., 2001; Zecca, Tampellini et al., 2001; Zucca et al., 2017). The loss of neuromelanin-containing dopaminergic neurons is responsible of Parkinson’s disease (PD), the second most common neurodegenerative disease that affects millions of people (Sveinbjörnsdóttir, 2016). Consequently, intensive research focused on the metabolic fate of dopamine over the past years.

The detailed pathway for neuromelanin biosynthesis is still obscure, but degradative studies show that, in SN, dopamine forms the predominant precursor and results in the production of mixed eumelanin and pheomelanin polymer (Ito, 2006; Wakamatsu, Fujikawa, Zucca, Zecca, & Ito, 2003; Wakamatsu, Murase, Zucca, Zecca, & Ito, 2012; Zecca, Gallorini et al., 2001; Zucca et al., 2014). This is in sharp contrast to the cutaneous melanin, which is biosynthesized from dopa without any participation from dopamine. Although a specific tyrosinase capable of oxidizing dopamine to dopamine quinone is lacking in the mammalian brain, enzymes such as prostaglandin H synthase, cytochrome P450, and brain peroxidases as well as iron could convert dopamine to dopamine quinone (Zucca et al., 2017). Glutathione and to some extent free cysteine form adducts with dopamine quinone. The resultant cysteinyldopamine after oxidative polymerization produces the pheomelanin pigment. The rest of the
dopamine quinone cyclizes intramolecularly generating leucodopa-
minechrome, which is rapidly oxidized to dopaminechrome (Pavlin,
Repic, Vianello, & Mavri, 2016). Isomerization of dopaminechrome
and the oxidative polymerization of resultant 5,6-dihydroxyindole
(DHI) lead to the production of eumelanin (Figure 1). Part of dopa-
mine quinone also reacts with external nucleophiles on proteins and
other cellular molecules. Packaging of eu- and pheomelanin with
aggregated proteins results in the deposition of melanin–protein ag-
gregates as components of neuromelanin in brain (Ito, 2006; Ito &
Wakamatsu, 2008; Wakamatsu et al., 2012; Zecca, Gallorini et al.,
2001; Zucca et al., 2014).

Dopamine is also essential for neural signaling in insects. In addi-
tion, it is associated with cuticular hardening (sclerotization), melanin
pigment production, camouflage, defense reactions, wound healing,
and thermoregulation (Barek, Sugumaran, Ito, & Wakamatsu, 2018;
Sugumaran, 2002, 2010; Sugumaran & Barek, 2016). Understandably,
dopamine is a central and indispensable molecule for the successful
survival of insect species. In insects, pigment production pathway
is intimately associated with cuticular sclerotization pathway. Dopamine
is primarily used for the production of the sclerotization precursors,
N-β-alanyldopamine (NBAD), and N-acetyldopamine (NADA) first. The
reactive species generated from these two compounds form adducts
and cross-links with cuticular proteins and chitin polymer resulting
in the hardening exoskeleton that protects all soft-bodied insects
(Sugumaran, 2010; Sugumaran & Barek, 2016). Several mutants asso-
ciated with the pigmentation pathway have paved way to identify the
intricate details of insect melanogenic pathway (Sugumaran & Barek,
2016). The pale gene codes for tyrosine hydroxylase activity. Dopa
thus formed is decarboxylated by dopa decarboxylase encoded by
Ddc gene. Ddc mutant flies have a soft and unpigmented cuticle. NBAD is
produced from dopamine by the action of NBAD synthetase encoded
by ebony gene. Ebony mutant flies have an increase in black coloration
in their cuticle due to the accumulation and use of dopamine for mela-
nin production. NADA is synthesized by N-acetyltransferase encoded

**FIGURE 1** A simplified model for the biosynthesis of eu- and pheomelanin portion of neuromelanin. Oxidation of dopamine generates
dopamine quinone. At this stage, the melanin pathway bifurcates. In the presence of cysteine, the pheomelanin pathway is promoted.
Thiols add on to dopamine quinone forming cysteinyldopamine derivatives. Oxidation of cysteinyldopamine to cysteinyldopamine quinone
and its oxidative polymerization lead to the production of pheomelanin pigment. When the cysteine content is low, eumelanin pathway
predominates. In this route, dopamine quinone undergoes intramolecular cyclization producing eventually dopaminechrome. Isomerization
of dopaminechrome to 5,6-dihydroxyindole, and subsequent oxidative polymerization of 5,6-dihydroxyindole leads to eumelanin. Cysteine
can also add on to indolequinone, and the resulting cysteinyld derivatives will lead to the formation of mixed eu- and pheomelanin pigment

**Significance**

*Drosophila* has served as an excellent model system for the
study of pathology and molecular biology associated with
a number of diseases. In Parkinson's disease, there is a sub-
stantial decrease in neuromelanin-containing dopaminer-
gen neurons. In order to examine whether insects have the
enzymatic machinery to make melanin portion of neu-
romelanin, proteins extracted from the larval brain of
*Drosophila melanogaster* were subjected to biochemical
analysis. Activity staining as well as mass spectral analysis
of the proteins separated on gel electrophoresis revealed
the presence of both dopachrome decarboxylase/tau-
tomerase and phenoloxidase, two of the major enzymes
associated with melanogenesis. Thus, *Drosophila* seems to
possess the enzymatic machinery to make melanin compo-
nent of neuromelanin.
β-Alanine needed for NBAD biosynthesis is produced by aspartate decarboxylase encoded by black. This mutant has the same phenotype as ebony mutant viz., increased black pigmentation in cuticle. Excess NBAD that is not used for sclerotization is converted back to dopamine by NBAD hydrolase encoded by tan and is consumed for cuticular melanin formation (Figure 2). The tan mutant flies have lighter colored cuticle due to their inability to produce dopamine needed for melanin biosynthesis. Thus, melanin production can be viewed as a sort of detoxification mechanism for the neutralization of excess dopamine, to avoid the deleterious reactions caused by the reactive species generated from NBAD (Sugumaran & Barek, 2016). This is similar to that reported for neuromelanin formation in dopamine neurons where excess cytosolic dopamine is utilized for neuromelanogenesis to prevent the action of toxic quinonoid products generated from dopamine (Sulzer et al., 2000). Therefore, one can equate the role of melanin production in the cuticle to that of neuromelanin in higher animals.

Several commonalities exist between neuromelanin biosynthesis and insect cuticular melanogenesis. First, dopamine is the major precursor for both neuromelanin and cuticular melanin (Sugumaran & Barek, 2016; Wakamatsu et al., 2003). Second, like neuromelanin, cuticular melanin is also made up of both eumelanin and pheomelanin (Barek et al., 2018; Galván, Jorge, Edelaar, & Wakamatsu, 2015). Third, both processes occur without the participation of melanosomes. Fourth, there are no signaling pathways associated with neuromelanin production. Similarly, cuticular melanin production after regeneration of dopamine from NBAD does not require input from any signaling pathway.

Interestingly, dopamine metabolism in the cuticle as well as insect brain follows an identical course. In Drosophila brain, dopamine is synthesized by the sequential action of tyrosine hydroxylase and dopamine decarboxylase on tyrosine, in a way that is very similar to how dopamine is made in the cuticle. Brain dopamine is packaged within synaptic vesicles via VMAT2 (encoded by Vmat) and is secreted by exocytosis at the presynaptic site upon depolarization of the neurons (Yamamota & Seto, 2014) to form neuromelanin. Dopamine then undergoes reuptake into the cytoplasm via the plasma membrane dopamine transporter (encoded by DAT). These reactions are conserved between higher animals and insects. The role of ebony, tan, and black in insects’ brain is similar to that in cuticle (dopamine recycling), with the important exception that NBAD and NADA are not used for sclerotization in brain. Some of the dopamine is converted into NBAD in glial cells. NBAD is then transported to the presynaptic neurons and is converted back to dopamine by the action of NBAD hydrolase (Figure 3). Consequently, ebony, tan, and black mutant flies not only exhibit defects in cuticular pigmentation but also show behavioral impairment (Yamamota & Seto, 2014). It should be pointed out here that all enzymes listed in Figure 3 are well characterized. But

**FIGURE 2** Dopamine metabolism for insect cuticle and pigmentation pathway. In the epidermal cell, tyrosine hydroxylase catalyzes the conversion of tyrosine into dopa encoded by pale. Dopa is then decarboxylated to dopamine by dopa decarboxylase (encoded by Ddc). Dopamine is primarily used for the biogenesis of the sclerotization precursors, NBAD and NADA, a reaction catalyzed by NBAD synthetase (encoded by ebony) and N-acetyldopamine transferase (encoded by aaNAT) respectively. Cuticular laccase (encoded by laccase 2) catalyzes the oxidation of NBAD and NADA to their corresponding quinones, which are used for sclerotization reactions that harden the insect cuticle. Unused NBAD is hydrolyzed back to dopamine by NBAD hydrolase (encoded by Tan). Dopamine thus regenerated is used for melanin biosynthesis. The mechanism of transport of NBAD, NADA, and dopamine from the epidermal cell into the cuticle has not yet been determined [Colour figure can be viewed at wileyonlinelibrary.com]
to best of our knowledge, there is no report on the characterization of melanogenic enzymes from any insect brain tissue.

From the foregoing discussion, it is clear that there exists a remarkable similarity between insect cuticular melanization and neuromelanin production observed in human, viz., melanin structure and precursor, role of the melanin formed, and the set of genes that are involved. It is also evident that insects use the same set of enzymes for the metabolism of dopamine in both their brains and cuticle with the exception of not using sclerotization reactions in the brain. Therefore, we hypothesized that insects might have the necessary enzymatic activities to biosynthesize melanic part of neuromelanin. The melanogenic enzymes found in cuticle and hemolymph are well characterized, but to best of our knowledge, no one has explored the biochemical activities of these enzymes in brain tissue. Hence, we conducted enzyme analysis of Drosophila larval brain tissue and our results indeed support the proposal that insects possess the enzymatic machinery to make melanin component of neuromelanin.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

The brain samples from 800 wild-type (Oregon R) Drosophila melanogaster were harvested from the third-instar larva by dissection. Brain tissues were stored until they are used at −80°C in 50 mM sodium phosphate buffer and 2 mM phenylthiourea to avoid the darkening of the sample. The brain tissue was homogenized, and soluble proteins were extracted with 50 mM sodium phosphate buffer pH 8.0. The extracted proteins were subjected to 0%–60% ammonium sulfate precipitation to isolate the enzymes associated with melanogenesis. The precipitated proteins were dissolved in water and subjected to dialysis against water. The dialyzed sample was subjected to native polyacrylamide gel electrophoresis on 4%–20% gels. After electrophoresis, the gels were washed repeatedly with distilled water and used for enzyme assays.

2.2 | Activity staining of phenoloxidase

Phenoloxidase exhibits wide substrate specificity. Apart from dopamine and other catecholamine derivatives, it also works very effectively with 4-methylcatechol producing its quinone. Our activity staining with dopamine failed to detect phenoloxidase as the staining was dependent on the amount of melanin formed. So we used a more sensitive technique involving the coupling of quinone formed from 2 mM 4-methylcatechol (10 ml) with 1 ml of 0.2% 3-methyl-2-benzothiazolinone hydrazone.HCl (MBTH). As one mole of quinone forms adduct with one mole of MBTH, the sensitivity of this assay is several orders of magnitude higher than that of the simple dopamine staining.
MBTH was first dissolved in ethanol, diluted with water (concentration of alcohol = 10%), and then used in the assay. This reaction produces a highly red-colored adduct and is highly sensitive to test the phenoloxidase activity (Sugumaran, 1998). Hence, we used this coupled assay of phenoloxidase for detecting this enzyme in brain tissue.

### 2.3 Activity staining of DCDT

For the detection of DCDT, same protocol was used as the above with the exception that proteins were extracted from 1000 brains of *Drosophila melanogaster* oreR. The gel was stained with dopaminechrome for 30 min to characterize dopachrome isomerase activity. Dopaminechrome was prepared by mixing 1.25 mM of dopamine with an equal volume of 2.5 mM of sodium periodate prepared in water. After the visualization of the band, dopaminechrome was discarded and the gel was washed repetitively with water (Nicklas & Sugumaran, 1995). The experiment was carried in triplicate, and the band containing the DCDT activity from one gel at about 250 kDa was subjected to mass spectrometry analysis.

### 3 RESULTS

#### 3.1 Detection of phenoloxidase activity

The first enzyme associated with melanogenesis in mammalian epidermis is tyrosinase, but in insects, two different closely related enzymes initiate melanogenesis by oxidizing catecholamine derivatives to their corresponding quinone products (Sugumaran, 2002; Sugumaran & Barek, 2016). O-Diphenoloxidase which specifically oxidize o-diphenols seems to be associated with hemolymph melanization, whereas as laccase, which can oxidize both o-diphenols and p-diphenols to their quinonoid products, is associated with cuticular sclerotization and melanization. In order to check phenoloxidase activity, protein extracts from *Drosophila* larval brain tissue were subjected to nondenaturing polyacrylamide gel electrophoresis and activity staining as outlined in an earlier publication (Sugumaran, 1998). The normal protocol with dopamine staining was not very sensitive to detect the enzyme present in the brain, but the protocol developed with 4-methylcatechol and MBTH is highly sensitive and allowed us to detect phenoloxidase in brain tissue. Using this protocol, we were able to show the presence of two phenoloxidase bands on the gel. The band of phenoloxidase that does not enter the gel corresponds to a highly polymerized insoluble form of the enzyme. Another faint band that is visualized around the molecular weight of about 250 kDa is the soluble enzyme form (Figure 4). Thus, phenoloxidase is present in the brain of *Drosophila melanogaster*.

#### 3.2 Detection of dopachrome decarboxylase/tautomerase activity

The next enzyme associated with insect melanogenesis is dopachrome decarboxylase/tautomerase (DCDT). *Drosophila* possesses a DCDT that converts dopachrome to DHI (Han et al., 2002). The same enzyme also catalyzes the conversion of dopaminechrome to DHI. To assess the presence of this enzyme, *Drosophila* brain homogenate was prepared and assayed for this enzyme activity. Incubation of brain protein extract with dopaminechrome resulted in the conversion of red-colored dopaminechrome to black-colored eumelanin pigment. Visible spectral analysis of the reaction also supported the ability of the protein extract to bleach dopaminechrome. Time course studies revealed a steady decrease in concentration of dopaminechrome due to its conversion to DHI. This enzyme preparation also acted on dopachrome (data not shown). To further confirm the presence of DCDT, we conducted specific activity staining as outlined in an earlier publication.
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with the exception of using dopaminechrome rather than dopachrome (Nicklas & Sugumaran, 1995). Protein extract was subjected to non-denaturing native polyacrylamide gel electrophoresis. A portion of the gel was subjected to activity staining with dopaminechrome, and another portion was stained with Coomassie Blue. Within 30 min, the gel treated with dopaminechrome exhibited a blue band at a molecular weight of about 250 kDa. This blue band eventually turned into a black band after 20 min (Figure 5). Similar to phenoloxidase, a polymeric band could also be visualized on top of the gel. These two bands thus correspond to DCDT activity present in the extracts.

The band corresponding to the DCDT on the Coomassie Blue-stained gel was cut out, digested with trypsin, and submitted to mass spectrometry analysis for protein identification. Comparison of the peptide fragments with the Drosophila protein data bank revealed a high number of proteins associated with this band. Pertinent to the current work is the presence of prophenoloxidase 1 and 2 (PPO1 and PPO2), which catalyze the oxidation of catecholamines to their corresponding quinones and yellow-f2 gene product viz., DCDT (Table 1). Taken together, both the mass spectral studies and activity staining experiments confirm the presence of both phenoloxidases and DCDT in Drosophila brain tissue.

3.3 Chemical analysis of Drosophila brain sample for melanin markers

Animals with a short lifespan do not seem to produce and accumulate neuromelanin in their brain (Zecca et al., 2006; Zucca et al., 2004, 2014). Nevertheless, in light of current findings viz., the detection of the melanogenic enzymes in the brain, we tried to see whether Drosophila larval brain contains neuromelanin by chemical analysis. The brain samples were digested and subjected to HPLC analysis to test for the presence of the eu- and pheomelanin markers (Ito & Wakamatsu, 1998, 2003; Wakamatsu, Ito, & Rees, 2002; Wakamatsu et al., 2003, 2012). The melanin markers were not detected. This would mean that either Drosophila does not make neuromelanin or the amount of neuromelanin present is below the detection limits of the instruments used to quantify melanin markers. Given the fact that out of 10,000 neurons found in the Drosophila larval brain, only 120 neurons are of dopaminergic type, it is understandable that detection of melanin in such a small amount of neurons would be difficult. It is also likely that animals with short lifespan will not make melanin in their brain. Regardless of these facts, the presence of prophenoloxidase activity as well as DCDT activity—two of the major proteins associated with the melanogenic pathway in the brain—indicates the feasibility of melanin production in Drosophila brain. Further studies are warranted to throw more light on this interesting aspect of catecholamine metabolism in insects.

4 DISCUSSION

Parkinson’s disease (PD) is a progressive neurological disorder that affects the life of mostly middle-aged people. The genes associated with PD as well as the modified metabolic pathways during the progression of the disease are well characterized (Blesa et al., 2015). One of the marked differences observed between healthy and PD
patients is the substantial reduction in neuromelanin-containing dopaminergic neurons. Therefore, several laboratories have extensively studied the biosynthesis and the physiological role of neuromelanin (Blesa et al., 2015; Ito, 2006; Wakamatsu et al., 2003, 2012; Zecca, Gallorini et al., 2001; Zecca, Tampellini et al., 2001; Zecca et al., 2006; Zucca et al., 2014, 2017). In spite of the substantial knowledge available on the synthesis, structure, bioaccumulation, physiological role in neuroprotection, and neurodegeneration of neuromelanin, many important details are still remained to be unraveled.

The genes implicated in PD appear to be highly conserved. Recently, several authors have indicated the usefulness of Drosophila to study PD (Lu & Vogel, 2009; Munoz-Soriano & Paricio, 2011; Yamamoto & Seto, 2014). The fruit fly, Drosophila melanogaster, has been an excellent tool to study the pathology and molecular biology associated with several diseases. Due to the important role of neuromelanin in PD, we assayed the protein extracts of Drosophila brain for melanogenic enzyme activities. The presence of such enzymes indicates that insects have the potential enzymatic machinery to make melanin part of neuromelanin although it has been reported that animals with short lifespan do not seem to make and accumulate neuromelanin in their brain (Zecca, Tampellini et al., 2001; Zecca et al., 2006; Zucca et al., 2014, 2017). In spite of the substantial knowledge available on the synthesis, structure, bioaccumulation, physiological role in neuroprotection, and neurodegeneration of neuromelanin, many important details are still remained to be unraveled.

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Mammalian system uses iron and oxidative enzymes other than tyrosinase to produce dopaminechrome (De Iuliis et al., 2008; Zucca et al., 2017). Although tyrosinase gene transcript and promoter activity have been detected in the human brain tissue (Tief, Schmidt, & Beerman, 1998), studies carried out with more reliable methods indicated that tyrosinase is not present in the human brain (Ikemoto et al., 1998; Tribl et al., 2006). In contrast, insect brain seems to possess phenoloxidase that is capable of oxidizing catechols to the quinonoid products. The activity of DCDT that converts dopachrome as well as dopaminechrome to DHI is also readily detected by the activity staining protocol as well as enzymatic assay analysis. The possible presence of a similar enzyme in mammalian system needs to be explored in the near future, especially as in vitro studies show that nonenzymatic conversion of dopaminechrome to DHI requires about 40 min, which is a long time for a reactive intermediate to be in the cell without causing any side reaction (Segura-Aguilar et al., 2014).

Based on our biochemical data and literature review, Figure 6 presents a model for dopamine metabolism in healthy and Parkinsonian brain. It is known that cytosolic dopamine that is not accumulated in the synaptic vesicles is converted to neuromelanin, which provides a protective role by trapping free radicals and reactive quinonoid intermediates, preventing oxidative stress (Herrera, Munoz, Steinnusch, & Segura-Aguilar, 2017; Sulzer et al., 2000). Cytosolic dopamine is
initially oxidized to dopamine quinone and is used for both eu- and pheomelanin production. The major fate of dopamine quinone appears to be through cyclization reaction that leads to eumelanin pigment, as the ratio of eu- to pheomelanin in neuromelanin is approximately 3:1 (Ito, 2006; Ito & Wakamatsu, 2008; Wakamatsu et al., 2012; Zecca, Gallorini et al., 2001; Zucca et al., 2014). The rest of the dopamine reacts with thiols generating cysteinyldopamine, which forms the precursor for pheomelanin or reacts with residues on aggregated proteins. We propose that dopaminechrome that is formed in the cytosol of dopaminergic neurons is isomerized to DHI by an enzyme-catalyzed reaction, much like the dopachrome tautomerase reaction that produces DHI-2-carboxylic acid in melanocytes. The presence of such a tautomerase will ensure the rapid conversion of dopaminechrome to DHI, which can be readily oxidized to eumelanin. Lack of this enzyme might result in the prolonged accumulation of dopaminechrome in the brain tissue. When the cell is unable to detoxify dopamine metabolites via eumelanin formation, pheomelanin production would become the sole pathway to absorb dopamine metabolites. This increased thiol consumption and pheomelanin production would result in rapid depletion of essential thiol pool. Consequently, dopamine metabolites tend to react with various proteins, which causes protein polymerization, proteasome dysfunction, and mitochondrial dysfunction (Segura-Aguilar et al., 2014; Zucca et al., 2017). Therefore, the possible presence of a tautomerase that might play a pivotal role in detoxifying the dopamine quinone metabolites needs to be examined.

The suggested model is in accordance with some of the observations made during the progression of PD. GSH level is reported to be significantly reduced in the SN of Parkinson's brain compared to age-matched controls (Ballatori et al., 2009; Pearce, Owen, Daniel, Jenner, & Marsden, 1997; Sian et al., 1994; Sofic, Lange, Jellinger, & Riederer, 1992). This depletion is apparently not accompanied by a corresponding increase in GSSG, nor is it caused by a decrease in the activity of the enzymes associated with the regeneration of GSH (Smeyne & Smeyne, 2013). The reactions of GSH with excess dopamine quinonoid metabolites would cause this effect (Ballatori et al., 2009). Accordingly, Shen, Li, and Dryhurst (2000)
and Fornstedt, Brun, Rosengren, and Carlsson (1989) observed an increase in the ratio of 5-S-cysteinyl dopamine to dopamine in the SN of parkinsonian patients during the progression of the disease and this increase paralleled the depigmentation and degeneration of SN neurons.

The root cause of PD is multifactorial, and various factors, such as genetic, aging, and environmental, contribute to the onset of PD. Alternate pathways that can lead to oxidative stress during the progression of PD have been proposed in the literature. Here, we propose that neuromelanin formation in any insect brain could be enzymatically controlled by a tautomerase that catalyzes the conversion of dopaminechrome to DHI. The absence of such an enzyme could be one of the factors contributing to the increased oxidative stress in the dopaminergic neurons. Further studies are necessary to shed more light on this aspect.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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