NADPH oxidase produces reactive oxygen species (ROS). *Drosophila melanogaster* has two homologs of NADPH oxidase, dNox and dDuox, with functions that remain unclear in vivo. To clarify these functions, two independent transgenic fly lines expressing dsRNA targeted for different portions of dDuox mRNA were used. In both flies, *en-GAL4* > UAS-dDuoxIR*976–1145* and *en-GAL4* > UAS-dDuoxIR*970–5119*, in which dDuox was knocked down selectively in the posterior area of the wing disc, the posterior compartment of the adult wings became paler and more fragile with wing veins that were indistinct by comparison with the anterior one. Fluorescence staining of the *en-GAL4* > UAS-dDuoxIR*976–1145* adult wings revealed that the ROS concentration in the posterior compartment was significantly lower than that in the anterior compartment. Moreover, in these flies, the posterior compartment of the wing imaginal disc showed a greater number of apoptotic cells detected by immunostaining with anti-cleaved caspase-3 antibody than those in the anterior compartment. Respective knockdown of tyrosine hydroxylase or dopa-decarboxylase showed paler wing blades in the posterior compartment similar to the phenotype of dDuox knockdown flies. Along with this observation, analysis of the catechol and dityrosine components in the wings of adult flies proved that dDuox plays important roles in the stabilization of the cuticle structure of the wings via tyrosine cross-linking, the sclerotization and melanization processes possibly through ROS production. These dDuox knockdown fly lines would be useful tools for further studying dDuox functions during the development of *Drosophila*.

During the 1990s, many lines of evidence demonstrated that reactive oxygen species (ROS)² were intentionally generated in diverse tissues by several members of NADPH oxidases identified in a wide variety of organisms, including mammals, nematodes, fruit flies, green plants, fungi, and slime molds (1). These enzymes contain a conserved Nox domain, including the NADPH-binding site at the COOH terminus, the FAD-binding site, six conserved transmembrane domains, and 4 highly conserved heme-binding histidines (2, 3). One special group of this family is named Duox (dual oxidase) for containing an additional peroxidase-like domain (4, 5). In humans, dual oxidases, hDuox1 and hDuox2, originally described as thyroid oxidases, were first discovered in the thyroid gland. A combination of several analytical methods showed that hDuox enzymes are also expressed in airway epithelial cells, although the mucosal surface of the trachea and the bronchi preferentially express hDuox1 (6). hDuox2 is located mainly at the apical membrane of enterocytes and in the brush border of the cecum and sigmoidal colon in the intestinal tract (7). Besides, these enzymes are present in a wide variety of tissues such as brain, cerebellum, mammary gland, muscle, nerve, placenta, and testis (8).

The NADPH oxidase family produces ROS that play important roles in many biological processes. When the phagocyte respiratory burst was discovered, Nox2-derived ROS were found to have a key role in host defense (2). However, under the control of a signaling network, dual oxidases produce ROS essential for killing microbes in normal host survival (6, 9, 10). Furthermore, ROS also inhibit phosphatases, activate kinases, and regulate ion channels and Ca²⁺ signaling (2). Protein oxidation by ROS provides a general form of signal that is used by many signaling pathways (3). In humans, Duox produces hydrogen peroxide required for the oxidation step of iodide in thyroid hormone production (8, 11). Additionally, Duox catalyzes the cross-linking of tyrosine residues in extra-cellular matrix modification (12, 13).

Despite the increasing importance of NADPH oxidase and ROS, their functions are not fully understood, especially those of Duox. Because of the wide distribution and high conservation across many organisms, the function of NADPH oxidase enzymes and ROS can be studied using simpler model organisms, such as *Drosophila melanogaster*. Only two NADPH oxidases, dNox and dDuox, exist in *Drosophila*. Both are highly homologous, but dDuox has an additional peroxidase domain that can produce H₂O₂. Both enzymes are expressed widely in various tissues such as head, eye, midgut, crop, heart, and ovary. Particularly, dDuox is abundantly expressed in the larval hindgut, trachea, and adult ovary (14). dNox was found to regulate agonist-induced calcium flux and smooth muscle contraction in ovarian tissue. Depletion of dNox resulted in a marked deficit in female egg-laying caused by a defect in ovulation (15). In addition, a signaling network composed of complex positive and negative mechanisms that control the expression and activity of dDuox to produce ROS was required for normal host survival in response to colonization of commensal or infectious microbes (9, 10, 16).
In the present study, the function of dDuox was further examined by selective knockdown of the dDuox gene in Drosophila by the GAL4-UAS expression system in combination with RNA interference. GAL4, a yeast transcriptional activator, can activate the transcription of any gene if the gene is preceded by a GAL4 upstream activating sequence (UAS) that consists of five optimized GAL4 binding sites (17). The GAL4 driver line of Drosophila contains the GAL4 gene downstream of the tissue-specific promoter. By crossing the GAL4 driver line with the fly line carrying the UAS-dDuoxIR gene, GAL4 proteins were expressed in specific cells and tissues of the progenies, which then activated the expression of the hairpin double-strand RNA of dDuox that led to the knockdown of the dDuox gene. The present work clarified that dDuox, possibly through ROS production, is required for normal wing development in Drosophila, particularly for the stabilization of the cuticle structure of the wings via the sclerotization, melanization, and tyrosine cross-linking processes.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks**—Fly stocks were maintained at 25 °C on standard food. The Canton S fly was used as the wild-type strain. The UAS-dDuoxIR976–1145 fly line was kindly provided by Dr. Won-Jae Lee, of Ewha Womans University (South Korea) (9). RNAi stocks 3308 and 3330 carrying inverted repeats of tyrosine hydroxylase (UAS-paleIR) and the dopa-decarboxylase gene (UAS-ddcIR), respectively, were obtained from the Vienna Drosophila RNAi Center. All other lines used in the present study, including GAL4 driver lines, were obtained from the stock center in Bloomington, Indiana.

**Oligonucleotides**—The following primers were used for constructing the UAS-dDuoxIR370–518 fly lines: dDuoxIR-F, 5′-AGTTGCTAGCATCCGGTTCTTTATCTGATGTC and dDuoxIR-R, 5′-GTGGCTAGCATCCGGTTCTTTATCTGATGTC. NheI sites are underlined. To obtain and clone an extracellular region of dDuox into pGEX6P-1 (GE Healthcare), the following PCR primers were synthesized from mRNAs of Canton S flies by using the SuperScript III One-Step RT-PCR System with Platinum® TaqDNA Polymerase (Invitrogen) and primers dDuoxF and dDuoxR. Then, the PCR product was digested with BglII and XhoI and inserted into the BamHI and XhoI sites of the pGEX-6P1 vector (GE Healthcare). Next, the obtained pGEX-dDuox was introduced into Escherichia coli BL21 to express GST-dDuox fusion proteins. Lysates of the E. coli BL21 cells were prepared by sonication in PBS containing 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation at 12,000 × g and 4 °C for 20 min and applied to a glutathione-Sepharose column (GE Healthcare). The column was washed with PBS containing 0.5 mM NaCl and 0.1% (v/v) Triton X-100 and then with a buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1 mM dithiothreitol. The adsorbed GST-dDuox fusion proteins were treated with Precision protease (GE Healthcare) for 16 h at 4 °C, and then dDuox protein was eluted with PBS.

**Production of Anti-dDuox Antibody**—The purified dDuox protein was used to elicit polyclonal antibody production in guinea pigs. Polyclonal antibodies reacting with dDuox were affinity-purified from the anti-serum of guinea pigs using HiTrap NHS (N-hydroxysuccinimide)-activated HP (GE Healthcare) coupled with GST-dDuox fusion protein after passage through GST-conjugated Sepharose HP.

**Establishment of Transgenic Flies**—P element-mediated germ line transformation was carried out by microinjection of the pWIZ-dDuoxIR370–518 vector into fertilized eggs as described in a previous study (19). F1 transformants were selected on the basis of white eye color rescue (19). This group has established seven independent transgenic lines carrying UAS-dDuoxIR370–518. Although essentially, the same results were obtained with these lines, line 14 carrying UAS-dDuoxIR370–518 on the third chromosome was used to double-check the phenotype of dDuox-knockdown flies.

**Knockdown of dDuox, Tyrosine Hydroxylase, and Dopadecarboxylase Genes in Drosophila**—UAS-dDuoxIR976–1145 transgenic flies were crossed with several GAL4 driver lines as listed in Table 1 and reared at 28 °C. The en-GAL4/Cyo-UAS-dDuoxIR976–1145 line was maintained and used for further testing. The second UAS-dDuoxIR370–518 transgenic flies were crossed with the en-GAL4 driver line to confirm the dDuox knockdown phenotype and exclude the potential off-target effects of dDuox dsRNA. The tyrosine hydroxylase or dopa-decarboxylase gene was knocked down by crossing UAS-paleIR or UAS-ddcIR with the en-GAL4 driver, respectively.

**Analysis of dDuox Protein Expression**—The dDuox gene was knocked down by leaky expression of the Hs-GAL4 driver at 25 °C. Whole extracts from third-instar larvae were electrophoretically separated on 6% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (GE Healthcare). The blotted proteins were detected with polyclonal antibodies against dDuox.
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Table 1

Summary of phenotypes induced by knockdown of dDuox with various GAL4 driver lines

| GAL4 line       | Expression of GAL4 (knockdown of dDuox) | Phenotype with UAS-dDuoxIR76–1145 |
|----------------|----------------------------------------|-----------------------------------|
| ActSC          | All tissue                             | Lethal                            |
| TubP           | All tissue                             | Lethal                            |
| Ey             | Eye disc                               | No detectable phenotype           |
| GMR            | Cells behind the morphogenetic furrow of eye disc | Dark circle                        |
| c179           | Embryonic mesoderm, larval muscles and wing imaginal discs | Pupa lethal                      |
| 5053A          | Embryonic longitudinal muscles, some cells in gut | No detectable phenotype           |
| En             | Posterior area of wing discs           | Held-out wings, indistinct wing veins, paler and fragile posterior compartments |
| Ve             | Wing disc, follicle cell, central nervous system and others | No detectable phenotype           |
| MS1096         | Dorsal wing disc                       | Held-out and fragile wings        |

Healthcare) in a solution containing 25 mm Tris-HCl, 190 mm glycine, and 20% (v/v) methanol for 4 h at 4 °C. Blotted membranes were blocked with TBS solution (20 mm Tris-HCl, pH 7.4, and 150 mm NaCl) containing 0.05% (v/v) Tween 20 and 5% (w/v) skim milk for 1 h at 25 °C. Then, membranes were incubated with guinea pig anti-dDuox antibodies produced in the present study at 1:1000 dilution, or mouse anti-α-tubulin monoclonal antibodies at 1:1000 dilution (Sigma) at 4 °C for 16 h. After washing with TBS containing 0.05% (v/v) Tween 20, the membranes were incubated with a horseradish peroxidase-labeled anti-guinea pig IgG (Rockland Immunochemicals, Inc.) or anti-mouse IgG (BioSource) at a 1:10,000 dilution for 2 h at 25 °C. Detections were performed with ECL Western blotting detection reagents (GE Healthcare). The images were analyzed by Lumivision ProHSII image analyzer (Aisin Seiki, Aichi, Japan).

Detection of apoptotic cells by immunohistochemistry—Third-instar larvae were dissected in PBS, and wing discs were fixed in 4% (w/v) paraformaldehyde in PBS for 30 min at 25 °C. After washing with 0.3% (v/v) Triton X-100 in PBS (PBST), samples were blocked for 30 min at 25 °C with 0.15% (v/v) Triton X-100 in PBS containing 1% (w/v) bovine serum albumin. Samples were then incubated with rabbit anti-α-tubulin monoclonal IgG (Cell Signaling Technology) at 1:100 dilution at 4 °C for 16 h. After extensive washing with PBST, the samples were incubated with goat anti-rabbit Alexa Fluor™ 488 (Molecular Probes, Invitrogen) at a 1:400 dilution for 2 h at 25 °C, further washed with PBST and PBS, and then mounted in Vectashield mounting medium (Vector Laboratories). Preparations were examined under a fluorescence BX-50 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K.K., Shizuoka, Japan).

In Vivo ROS Detection—Adult flies from newly hatched en-GAL4/++;UAS-dDuoxIR76–1145/+ flies were dissected in PBS and then incubated with 10 μM 5- (and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Molecular Probes, Invitrogen) for 5 min. After washing three times with PBS, samples were mounted in Vectashield mounting medium (Vector Laboratories). Preparations were examined under a fluorescence BX-50 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K.K., Shizuoka, Japan).

Analysis of Amino Acid Components in Wings—Wings of 3-day-old flies were hydrolyzed by 6 M HCl in evacuated test-tubes at 110 °C for 24 h. The hydrolytic products from 5 adult wings were dried and dissolved in 300 μl of amino acid analyzer buffers (pH 2.2). 50 μl of these samples were analyzed for amino acid composition and concentrations using an amino acid analyzer (Hitachi, model L-8500A, Tokyo, Japan). The experiments were repeated three times.

Analysis of Dityrosine Content—Wings of 3-day-old flies were hydrolyzed under the same conditions described in the preceding paragraph. The hydrolysate of 15 adult wings were dried and dissolved in 1200 μl of PBS. Then, 100 μl samples were coated on wells and left overnight at 4 °C. After coating and washing with PBS solution containing 0.05% (v/v) Tween 20 (TPBS), the plate was blocked with 5% (w/v) nonfat dry milk in PBS for 1 h at 25 °C and then incubated with a monoclonal antibody against dityrosine, which was kindly provided by Dr. Yoji Kato (University of Hyogo), at 1:200 dilution (5 μg/ml) for 1.5 h at 25 °C (20). After washing five times with TPBS, the wells were incubated with a horseradish peroxidase-labeled anti-mouse IgG (BioSource) at 1:5000 dilution for 1.5 h at 25 °C. Color development was performed by reaction of the conjugated peroxidase with 0.4 mg/ml o-phenylenediamine dihydrochloride (Sigma) and H2O2 (Wako). Absorbance at 492 nm was measured by a microplate reader (MTP-120, Corona Electric). Wells without a coating step were used as a negative control. The experiment was repeated three times.

Colorimetric Determination of Catechols—Hydrolitic products from 40 adult wings were dissolved in 200 μl of 1 N HCl, 50 μl of which was analyzed for catechols by reacting with ammonium-iron citrate and 1,10-phenanthroline (21). All of the reagent solutions were prepared daily. The total volume of the assay was decreased to 250 μl for application to a microplate scale. 200 μM 1,3,4-dihydroxyphenylalanine (l-Dopa) in 0.1 N H2SO4 was used as a positive control. Absorbance at 492 nm was measured using a microplate reader (MTP-120, Corona Electric, Japan). The experiment was performed in triplicate each time and repeated three times.

Statistical Analysis—The results are expressed as means ± S.D. The statistical significance of the differences was evaluated using a t test for two samples assuming either equal or unequal variances. Values of p < 0.05 were considered significant.

RESULTS

Effect of Tissue-specific Knockdown of dDuox on Phenotype—The present study crossed the UAS-dDuoxIR76–1145 fly line with several GAL4 driver lines that express GAL4 selectively in various tissues to investigate the function of dDuox. As summarized in Table 1, knockdown of dDuox in the whole bodies of the flies by Act5C-GAL4 and Tub-GAL4 or in the mesoderm, muscles, and wing discs by c179-GAL4 caused lethal effects suggesting that the dDuox gene is essential to the normal develop-
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Knockdown of Duox Disrupts Normal Wing Development

Because the phenotype was easily recognizable, the present study focused on the wing phenotype of Duox-knockdown flies by the en-GAL4 driver for further experiments. The flies carrying a single copy of both the en-GAL4 driver and UAS-dDuoxIR76–1145 (en-GAL4/+;UAS-dDuoxIR76–1145/+), showed the held-out wings phenotype with an indistinct wing veins in the posterior compartments (Fig. 1, b and b’). Compared with those of the control flies containing one copy of en-GAL4 only (en-GAL4/+;+, Fig. 1, a and a’), the colors of the posterior compartments of these flies were paler than the anterior ones, probably due to less pigmentation in the posterior area. These effects may be caused by the disruption of some developmental processes of the wings, including wing pigmentation.

A BLAST search was used to verify that the dsRNA of the Duox RNAi gene in UAS-dDuoxIR76–1145 had no significant perfect matches in 19–21 nucleotides of other sequences in the fly genome, thereby eliminating potential problems with cross-silencing (9). To further exclude possible off-target effects, the UAS-dDuoxIR370–518 line, into which the RNAi gene corresponding to amino acids from 370 to 518 was introduced, was constructed. The second-independent Duox-knockdown flies were established by crossing with the en-GAL4 driver. The phenotype of flies en-GAL4/+;UAS-dDuoxIR370–518/+ was essentially the same as that of the flies carrying a single copy of both the en-GAL4 driver and UAS-dDuoxIR76–1145 (en-GAL4/+;UAS-dDuoxIR76–1145/+), indicating that the effects were truly caused by specific knockdown of the Duox gene.

The age-dependant change of wing morphology was examined for 20 flies from each group: the control (en-GAL4;+) and Duox-knockdown flies (en-GAL4;UAS-dDuoxIR76–1145). In the Duox-knockdown flies, en-GAL4/UAS-dDuoxIR76–1145, wings were damaged age-dependently: 1 day (a and a’); 3 days (b and b’); and 6 days (c and c’) after hatching, whereas no damage was observed in the control group 6 days after hatching (d and d’). The dashed circles indicate damaged areas of the wings. Mounted wing samples are in the lower panel (a’ to d’). Flies were reared at 25 °C.

FIGURE 1. Knockdown of dDuox disrupts normal wing development. Adult wings of the control flies en-GAL4/++;+ (a and a’); dDuox-knockdown flies en-GAL4/++;UAS-dDuoxIR76–1145/++;+ (b and b’); and another independent dDuox-knockdown flies, en-GAL4/++;UAS-dDuoxIR76–1145/++;+, have essentially the same dDuox-knockdown phenotype (c and c’). The dDuox-knockdown-induced wing phenotype was suppressed by deletion of reaper (rpr), head involution defective (hid), and grim when they were heterozygous in en-GAL4/++;UAS-dDuoxIR76–1145/Dr(3L)H99 (d and d’). Flies were reared at 28 °C.

FIGURE 2. Age-dependent dDuox-knockdown-induced phenotype. The morphology change by age was observed for 20 flies from each group: the control (en-GAL4;+) and dDuox-knockdown flies (en-GAL4/++;UAS-dDuoxIR76–1145). In the Duox-knockdown flies, en-GAL4/UAS-dDuoxIR76–1145, wings were damaged age-dependently: 1 day (a and a’); 3 days (b and b’); and 6 days (c and c’) after hatching, whereas no damage was observed in the control group 6 days after hatching (d and d’). The dashed circles indicate damaged areas of the wings. Mounted wing samples are in the lower panel (a’ to d’). Flies were reared at 25 °C.

Development of Drosophila. The abnormal phenotype on the wings was observed in adults by knockdown of the Drosophila gene with en-GAL4 and MS1096-GAL4 drivers. The compound eyes of flies in which the dDuox gene was knocked down by the GMR-GAL4 driver exhibited dark circles inside ommatidia (Fig. S1). However, a knockdown of dDuox by ey-GAL4, 5053-GAL4, and ve-GAL4 drivers showed no detectable phenotypes. This is possibly because of a low level of GAL4 protein expressed under the control of these promoters, which led to the insufficient expression of dDuox dsRNA in these flies.

Knockdown of dDuox Disrupts Normal Wing Development—Because the phenotype was easily recognizable, the present study focused on the wing phenotype of dDuox-knockdown flies by the en-GAL4 driver for further experiments. The flies carrying a single copy of both the en-GAL4 driver and UAS-dDuoxIR76–1145 (en-GAL4/+;UAS-dDuoxIR76–1145/+), showed the held-out wings phenotype with an indistinct wing venation in the posterior compartments (Fig. 1, b and b’). Compared with those of the control flies containing one copy of en-GAL4 only (en-GAL4/+;+, Fig. 1, a and a’). Moreover, the colors of the posterior compartments of these flies were paler than the anterior ones, probably due to less pigmentation in the posterior area. These effects may be caused by the disruption of some developmental processes of the wings, including wing pigmentation.
Knockdown of the dDuox gene affected dDuox protein levels—To confirm that dsRNA_976–1145 silenced the expression of the dDuox gene, the dDuox protein level was analyzed by Western immunoblot using anti-dDuox polyclonal antibody produced in guinea pigs. Anti-α-tubulin antibody detected bands at ~55 kDa confirming the equivalent protein loading between the extracts of Canton S (wild-type) and the leaky knockdown strain (Hs-GAL4/++; UAS-dDuoxIR_976–1145/+ flies) (Fig. 3). With anti-dDuox antibody, the expected 180 kDa band was detected in the larval extracts from Canton S as a single band. The band was significantly decreased by 72% in extracts from Hs-GAL4/++; UAS-dDuoxIR_976–1145/++, indicating that dsRNA_976–1145 had successfully silenced the expression of the dDuox gene. The results also indicated the high specificity of anti-dDuox antibody.

dDuox Produced ROS in Wings—It is known that Duox has an important role in producing ROS, especially H2O2 (6). Therefore, to investigate the activity of dDuox enzyme in knockdown flies, adult wings of en-GAL4/++; UAS-dDuoxIR_976–1145/+ were reacted with a non-fluorescent substrate, CM-H2DCFDA, which can be oxidized by ROS to generate an intracellular green fluorescent product. The ROS signal was quite strong in the anterior compartment of the adult wing, whereas there was no detectable signal in the posterior compartment in which dDuox dsRNA was specifically expressed (Fig. 4, b and b’). In contrast, adult wings of the control flies (en-GAL4/++; +) showed strong fluorescent signals in both the posterior and anterior compartments (Fig. 4, a and a’). These results suggested that dDuox functions to produce ROS in wings, and ROS production was suppressed by dDuox-knockdown.

Knockdown of dDuox Led to Increased Cell Death—The observation that dDuox-knockdown flies had fragile wing blades suggested the possible involvement of an apoptosis process. Therefore, the third instar larval wing discs of the control and the dDuox-knockdown flies were immunostained with anti-cleaved caspase-3 IgG to detect caspase-dependent apoptosis (Fig. 5, d and e). The posterior compartment of the wing imaginal disc in which dDuox was knocked down by the en-GAL4 driver exhibited increased apoptotic cells compared with those of control flies. As shown in Fig. 5g, the difference in apoptotic cell number in the posterior areas between dDuox-knockdown flies and control flies was statistically significant (p < 0.05, Student’s t test), whereas that in the anterior areas was not. These results indicated that knockdown of dDuox in the wing disc induced apoptosis.

In Drosophila, three positive regulators of apoptosis have been identified. These genes, reaper (rpr), head involution defective (hid), and grim, all lie at the locus 75C1-2 (22). The dDuox-knockdown flies en-GAL4/CyO; UAS-dDuoxIR_976–1145 were crossed with the chromosome deficiency line Df(3L)H99 in which these three genes had been deleted. The dDuox-knockdown wing phenotype in heterozygous progenies (Fig. 1, d and d’) were suppressed effectively and showed nearly the same phenotype as the control flies (Fig. 1, a and a’). In the posterior compartment of the imaginal wing discs in heterozygous progenies, apoptotic cells were clearly reduced (Fig. 5f) compared with those of the knockdown flies (en-GAL4/CyO; UAS-dDuoxIR_976–1145/H99) (Fig. 5e). As shown in Fig. 5g, the difference in apoptotic cell number in posterior areas between dDuox-knockdown flies and en-GAL4/CyO; UAS-dDuoxIR_976–1145/H99 flies was statistically significant (p < 0.05, Student’s t test). Therefore, the knockdown of dDuox induced an apoptotic process in wing imaginal discs that partially caused the adult wing phenotype.

dDuox Was Involved in Stabilization of Cuticle Structure—The posterior compartments of dDuox-knockdown adult wings were fragile and paler in color compared with the ante-
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knocked down by the en-GAL4 driver to examine the effects on adult wing phenotypes (Fig. 6, b and c, respectively). These knockdown flies also showed paler wing blades in the posterior compartment similar to the phenotype of dDuox-knockdown flies (Fig. 6a). However, in contrast to dDuox-knockdown flies, pale- and Ddc-knockdown flies did not show the held-out wing phenotype.

The amino acid compositions of adult wings of dDuox-knockdown flies (en-GAL4/CyO;UAS-dDuoxIR976–1145) and the control flies (en-GAL4/CyO) were consistently identical (Fig. 6d). The average amount of amino acids in each adult wing of the control flies and dDuox-knockdown flies were not significantly different, showing 8.6 and 7.9 nmol/wing, respectively (p > 0.05, Student’s t test). Therefore, equal numbers of wings from adult knockdown flies and control flies were used after hydrolysis for quantifying catechol compounds, which are important precursors in cuticular sclerotization and melanization. L-Dopa was used as the positive control. The catechol component in the dDuox-knockdown wings was lower than that of the control sample (Fig. 6e). The differences were statistically significant (p < 0.05, Student’s t test). These results indicated that the disturbance of normal cuticular sclerotization and melanization in wing development was one of the effects caused by down-regulation of the dDuox enzyme.

Recently, the homolog of the Duox gene in Caenorhabditis elegans (Ce-Duox) was proved to catalyze cross-linking between collagen and other proteins via di- and trityrosine linkages to stabilize the cuticular extracellular matrix (12). Therefore, the dityrosine component in hydrolysis samples of wings of the dDuox-knockdown and the control flies was analyzed further (Fig. 6f). The negative control wells showed a low background signal. In the control samples (hydrolysis sample of wings of en-GAL4/CyO flies), the absorbance was much higher than that of the dDuox-knockdown samples (hydrolysis sample of wings of the dDuox-knockdown flies). Therefore, knockdown of the dDuox enzyme decreased the formation of dityrosine linkages, which very likely affected the stabilization of the cuticle structure of the wings in Drosophila.

DISCUSSION

Recently, many lines of evidence have proved that ROS are intentionally produced by members of the NADPH oxidase family, and ROS play important roles in several biological processes, including host defense, hormone biosynthesis, fertilization, and cellular signaling (10, 12, 13, 15). However, in vivo functions of this oxidase family and the derived-ROS are limited.

In the present study, knockdown of the dDuox gene in the whole body of D. melanogaster was lethal, indicating its essential functions. Selective knockdown of two independent dDuoxIR flies by en-GAL4 caused the held-out wing phenotype with an indistinct wing veins and a paler and more fragile posterior compartment compared with the anterior one. Using the MS1096-GAL4 driver also caused a similar knockdown phenotype in the wing blade. However, when dDuox was knocked down by a ve-GAL4 driver, no detectable wing phenotype was observed, even though the ve-GAL4 driver functions in tissues...
that includes the wing disc. This is possibly due to the insufficient expression of dDuox dsRNA by this GAL4 driver.

Previously, Xie and co-workers (24) reported that the ubiquitous knockdown of nip encoding the dDuox maturation factor, NIP/DuoxA, in Drosophila using a daG32-GAL4 driver led to abnormally crinkled wings. When combined with their results, the present study could confirm the importance of the role of the dDuox system in wing development. Xie et al. (24) also indicated that ubiquitous nip-knockdown reduced the lifespan at 29 °C as well as the ability to survive under oxidative stress and displayed an impaired mitochondrial aconitase function. Therefore, dDuox-knockdown flies may have the same effects as NIP/DuoxA-knockdown. However, we cannot evaluate this, because in our experiments, dDuox was knocked down using an en-GAL4 driver. With this GAL4 driver, the knockdown is restricted in the posterior area of wing discs and therefore has minor impact on the lifespan and oxidative stress response of the entire body. In addition, in our experiments, ubiquitous knockdown of dDuox by Act5C-GAL4 and Tub-GAL4 resulted in lethal effects as indicated in Table 1.

H₂O₂, a major ROS produced by dDuox, is known as a cell survival signaling molecule at low concentrations (25). Therefore, decreased cell survival signaling of ROS might cause an increase of apoptotic cells in the posterior compartment of the wing in dDuox-knockdown flies. However, adult dDuox-knockdown flies have a normal wing size, probably as a result of compensatory proliferation (26). Besides, strong suppression of the dDuox-knockdown-induced wing phenotype was observed by genetic crossing with Df(3L)H99 flies in which genes encoding three regulators of apoptosis had been deleted. These observations might imply that the specific cells that undergo apoptosis in the wings of dDuox-knockdown flies are the major producer of ROS that are involved in wing development. Another possibility is that unidentified signaling pathways following the apoptosis processes might partially cause the dDuox-knockdown phenotype. In any event, these results indicate that the dDuox-knockdown-induced wing phenotype was at least partially caused by an apo-

![Figure 6](image-url)

**FIGURE 6.** dDuox has roles in the stabilization of cuticle structure. Compared with dDuox-knockdown flies (a), knockdown of pale (b) and Ddc (c) genes results in a similar paler posterior compartment phenotype. a, X/X;en-GAL4/++;UAS-dDuoxIR976–1145/++; knockdown of dDuox; b, UAS-DdcIR/X;en-GAL4/++;+, knockdown of Ddc; c, X/X;en-GAL4/UAS-paleRi/+; knockdown of pale. The posterior areas of the wings are on the downward side. Flies were reared at 28 °C. Amino acid compositions of adult wings (nmol/wing) (d). The hydrolysis products of adult wings were used in colorimetric determination of catechol components (e) and analysis of dityrosine component by ELISA with monoclonal antibody against dityrosine (f). Data are expressed as the mean ± S.D. The statistical significance of the differences between samples from dDuox-knockdown flies and control flies was evaluated using a t test for two samples assuming equal variances. *, values of p < 0.05 were considered significant.
ptosis process, possibly through some signaling pathways related to ROS.

Additionally, in the present study, it was assumed that dDuox-knockdown flies might have some defect in the sclerotization and melanization processes of wings in which the cuticle is stabilized and tanned by incorporation of phenolic compounds (23). Tyrosine hydroxylase and dopa-decarboxylase are known for their key roles in these processes (23, 27). Subsequently, when each of the genes encoding for these two enzymes, the pale or ddc gene, was knocked down using the en-GAL4 driver, the flies had a paler posterior compartment of their wings similar to dDuox-knockdown flies, suggesting the involvement of dDuox in firm wing formation. Quantitative analysis indicated that the catechol content of the wings of dDuox-knockdown flies was less than that of the control flies. This result also suggested that ROS produced by dDuox may involve in the sclerotization and melanization of the wing. However, because pale- and ddc-knockdown flies did not exhibit the held-out wing phenotype, dDuox may also be involved in other processes besides sclerotization and melanization.

Recently, the homolog of the Duox gene in C. elegans was proven to catalyze cross-linking between collagen and other proteins via di- and trityrosine linkages that stabilize the cuticular extracellular matrix (12). The present research also showed a lower dityrosine component in the wings of dDuox-knockdown flies that may lead to more fragile wings compared with the control flies. Besides, tyrosine cross-linkages were known for stabilizing many proteins, particularly resilin, which account for 80% of the wing ligaments. This may also explain the unusual held-out wing phenotype of the dDuox-knockdown flies.

In conclusion, ROS generated by dDuox may involve in the formation of a tyrosine linkage, sclerotization, and melanization of the cuticle, which we believe may explain the held-out wing phenotype, and a paler, more fragile posterior compartment with indistinct wing veins of dDuox-knockdown adult flies. However, further study is needed to clarify the involvement of apoptosis in wing development. The transgenic flies and antibodies developed in the present study can provide useful tools for the further study of dDuox functions during the development of Drosophila.

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