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Heterozygous Mutation of Opa1 in Drosophila Shortens Lifespan Mediated through Increased Reactive Oxygen Species Production

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Abstract

Optic atrophy 1 (OPA1) is a dynamin-like GTPase located in the inner mitochondrial membrane and mutations in OPA1 are associated with autosomal dominant optic atrophy (DOA). OPA1 plays important roles in mitochondrial fusion, cristae remodeling and apoptosis. Our previous study showed that dOpa1 mutation caused elevated reactive oxygen species (ROS) production and resulted in damage and death of the cone and pigment cells in Drosophila eyes. Since ROS-induced oxidative damage to the cells is one of the primary causes of aging, in this study, we examined the effects of heterozygous dOpa1 mutation on the lifespan. We found that heterozygous dOpa1 mutation caused shortened lifespan, increased susceptibility to oxidative stress and elevated production of ROS in the whole Drosophila. Antioxidant treatment partially restored lifespan in the male dOpa1 mutants, but had no effects in the females. Heterozygous dOpa1 mutation caused an impairment of respiratory chain complex activities, especially complexes II and III, and reversible decreased aconitase activity. Heterozygous dOpa1 mutation is also associated with irregular and dysmorphic mitochondria in the muscle. Our results, for the first time, demonstrate the important role of OPA1 in aging and lifespan, which is most likely mediated through augmented ROS production.

Introduction

OPA1 is a ubiquitously expressed large dynamin-related GTPase, encoded by the nuclear genome and targeted to the inner mitochondrial membrane [1–3]. The predicted structure of OPA1 reveals a mitochondrial targeting signal, a transmembrane domain, a presenilin-associated rhomboid-like protease (PARL) recognition site, and a dynamin/GTPase domain. The mitochondrial inner membrane PARL is required for the correct proteolytical processing of OPA1 and subsequent assembly of OPA1 oligomers [4]. OPA1 promotes mitochondrial fusion and is essential for maintaining normal mitochondrial morphology. In addition, OPA1 regulates cytochrome c mediated apoptosis by modulating mitochondrial cristae structures [5,6].

In human, mutations of OPA1 cause dominant optic atrophy (DOA), the most common form of autosomal inherited optic neuropathy. DOA is characterized by degeneration of retinal ganglion cells [7] and progressive loss of vision [8]. Optical atrophy can be accompanied by deafness and/or neurological manifestations [9–12]. The most prevalent form of DOA is caused by mutations in the OPA1 protein [13]. Although a substantial progress has been made to unravel the molecular function of OPA1, the pathogenesis of DOA remains poorly understood. Recently, we have generated a Drosophila knockout model for optic atrophy [14]. Heterozygous mutation of dOpa1 by a P-element or transposon insertions caused no discernable eye phenotype under a light microscope, whereas the homozygous mutation resulted in embryonic lethality. In the eye-specific somatic clones, the somatic homozygous mutation of dOpa1 in the eyes caused rough (mis patterning) and glossy (decreased lens and pigment deposition) eye phenotypes in adult Drosophila. In Drosophila eyes, dOpa1 mutation resulted in elevated ROS generation and mitochondrial fragmentation associated with damage and death of the cone and pigment cells. Treatment with antioxidants or superoxide dismutase (SOD1), as well as over-expression of human SOD1 did reverse the glossy phenotype, further indicating the important role of ROS in etiology of optic atrophy.

ROS remain as one of the most widely accepted cause of aging. Mitochondrial dysfunction was shown to affect longevity [4,15]. Aging in biological systems is also accompanied by mitochondrial morphological changes [13]. Reducing mitochondrial fission in two fungal models resulted in decreased mitochondrial fragmentation and extended lifespan by increased resistance to induction of apoptosis [16]. Since OPA1 is ubiquitously expressed and OPA1
mutation is associated with altered mitochondrial dynamics and elevated ROS production in the Drosophila retinal cells, OPA1 insufficiency may also accelerate aging and affect lifespan. In this study, we further exploited our Drosophila model to investigate the effects of dOpa1 mutation on ROS production in Drosophila, mitochondrial complex activities and lifespan. We found that heterozygous mutation of dOpa1 results in a shortened lifespan, an increased production of ROS, sensitivity to oxidative stress, defects in complex activities of respiratory chain and aberrant mitochondrial morphology. Our studies suggest that heterozygous mutation of dOpa1 causes shortened lifespan mediated through increased ROS production.

Results

Heterozygous mutation of dOpa1 results in decreased lifespan in Drosophila

To test the hypothesis that heterozygous mutation of dOpa1 affects the lifespan in Drosophila, we performed a longevity assay on a large cohort (n = 300/genotype) of dOpa1+/− (dOpa1+/ins, dOpa1+/ex2, and dOpa1+/ex+), and dOpa1+/+ control Drosophila. We previously showed that the P-element insertion in exon 2 of dOpa1+/ex2, and transposon insertion in intron 3 (dOpa1+/ ins) disrupted dOpa1 expression while insertion in non-coding exon 14 had no effect on the dOpa1 protein level, therefore, dOpa1+/ex14 also served as a control. Drosophila were maintained at 40-50 Drosophila/vial, transferred to fresh food and counted every 3 days. As shown in Figure 1, the dOpa1+/− mutant strain had a significant reduction in both average and maximum lifespan. Since there is no phenotypical difference between dOpa1+/ins and dOpa1+/ex2, dOpa1+/ins is referred as dOpa1+/− in all subsequent experiments. Wild-type Drosophila with the same background are used as the control and referred as dOpa1+/++. 

Heterozygous mutation of dOpa1 results in decreased resistance to oxidative stress and increased reactive oxygen species (ROS)

ROS damage biological macromolecules and have been shown to be a major cause of aging. In a previous study, we found that somatically generated homozygous dOpa1 mutation large clones displayed higher ROS levels in dOpa1+/− cells than in dOpa1+/− cells in the eyes [14]. In this experiment, we used MitoSOX to measure the ROS levels of 7-day old dOpa1+/− and dOpa1+/− whole flies, and observed that both ROS levels and generation rates were significantly elevated in dOpa1+/− Drosophila (Figure 2A, B).

One important genetic determinant for the lifespan of an organism is its sensitivity to oxidative stress [17]. Paraquat can generate more superoxide anion that can lead to synthesis of more ROS [18]. Susceptibility to paraquat can indicate the tolerance of the organism to oxidative stress. To test the overall fitness of dOpa1+/− compared to dOpa1+/++ Drosophila, we exposed adult flies to paraquat for a prolonged period of time and monitored their survival. Compared to control animals, dOpa1+/− Drosophila exhibited a significantly increased sensitivity to oxidative stress (Figure 2C).

Heterozygous mutation of dOpa1 leads to a respiratory defect in Complex II and III of the electron transport chain (ETC)

In order to further investigate the mechanism by which dOpa1 mutation elevates ROS, we performed a detailed comparison of respiration of the mitochondria of dOpa1+/− and dOpa1+/− adult Drosophila (7 d.o.), as inhibition of the mitochondrial ETC can increase ROS. In oxygen consumption assays, metabolism of NADH-linked complex I substrates pyruvate and malate was unaffected by dOpa1+/− mutation while succinate-driven respiration via complex II was significantly decreased (P<0.05) in dOpa1+/− Drosophila (Figure 3A). Consistent with these findings, while no significant differences were observed in the specific activities of complexes I and IV in 7-day old dOpa1+/− and dOpa1+/− flies, complex II (39% reduction, p = 0.001) and III (37% reduction, p = 0.026) activities were significantly attenuated in dOpa1+/− Drosophila (Figure 3B). In 35 d.o. dOpa1+/− flies, a similar impairment of enzymatic activities in complexes II (37% decrease, p = 1.97e-07) and III (28% decrease, p = 0.008) was observed. In addition, moderate (10%), but significant (p = 0.02) decline of complex IV activity was observed in 35 d.o. dOpa1+/− flies (Figure 3C). Our result also showed that there was a gender difference in the complex II activity. Heterozygous mutation of dOpa1 in males caused more significant inhibition of the complex II activity at 5 week of age (Supplemental Figure S1).

Mitochondria are not only the major sources of ROS production, but the complexes are also vulnerable targets of ROS [19]. To investigate whether the decreased complex activities cause increased ROS production or increased ROS inhibits the complex activities in the dOpa1+/− mutant flies, we studied their mitochondrial aconitase activity. In the 7-day old flies, no significant differences in mitochondrial aconitase activities were observed between dOpa1+/− and dOpa1+/− flies (data not shown). However, in the 35-day old dOpa1+/− flies, mitochondrial aconitase activities were reduced by 22% relative to age-matched controls (Figure 3D). Furthermore, after reactivation with dithiothreitol and iron, aconitase activity was restored to an even higher level in dOpa1+/− flies. This result suggests that ETC dysfunction is the primary cause. Since the inhibition of aconitase activity is only observed in the older flies and the inhibition was reversible, increased ROS may enforce a vicious cycle and further lead to mitochondrial dysfunction.

The shortened lifespan of heterozygous dOpa1 mutants is extendable through the administration of antioxidants in male flies, but not in female flies

Exogenously supplemented antioxidant can detoxify ROS and may retard aging. We have shown that antioxidant treatments were able to ameliorate ROS damage-induced glossy eye phenotype in dOpa1 mutant large clones [14]. To study if antioxidant administration can reverse the observed reduced lifespan of the dOpa1+/− mutant strain, we added MnTBAP to the fly food and generated the survival curves for dOpa1+/− flies. Since males and females of the same species may differ in their responses to interventions that affect lifespan [20], we separated male and female flies in the survival study. Indeed we observed sex-specific effects of antioxidant on lifespan in dOpa1+/− flies. While MnTBAP treatment showed no consequences on lifespan of female dOpa1+/− flies, significantly increased lifespan (50% survival time increased by 7 days) was observed in antioxidant-fed male dOpa1+/− flies (Figure 4).

Heterozygous mutation of dOpa1 results in highly irregular and dysmorphic mitochondria in the skeletal muscle of adult Drosophila

We previously described morphologically perturbed mitochondria in dOpa1+/− cells of ‘large clone’ Drosophila compound eyes [14]. Since OPA1 is ubiquitously expressed, it is interesting to investigate if dOpa1 mutation also leads to morphological aberrancy of mitochondria in other organs than the eyes. We
used TEM analysis (Figure 5) and found that the dOpa1+/2 mitochondria were highly irregular and dysmorphic (Left) compared to dOpa1+/+ mitochondria (Right) in the muscle. This result suggests that heterozygous mutation of dOpa1 also affects the mitochondrial dynamics in Drosophila.

Discussion

OPA1 is involved in mitochondrial fusion, cristae organization, control of apoptosis, ROS production and mutations in OPA1 genes lead to DOA. Using the Drosophila model, we demonstrated that heterozygous mutation of dOpa1 resulted in a shortened lifespan, enhanced production of ROS and boosted sensitivity to oxidative stress. Impairment of respiratory chain complex activities and age-dependent defects in aconitase activity were also observed, which is associated with irregular and dysmorphic mitochondria in the skeletal muscle. In male dOpa1+/2 flies, shortened lifespan can be increased by administration of antioxidants.

Mitochondrial energy metabolism and ROS toxicity are important factors affecting Drosophila aging. The Indy mutant restricting the availability of calories lived longer [21]. Transgenic flies expressing increased Cu/ZnSOD and catalase [22] displayed extended lifespan. OPA1 is an important regulator of mitochondrial fusion and provide protection for the mitochondria from damage. OPA1 mutations in DOA patients have been associated with mitochondrial fragmentation [23,24], mtDNA copy number reduction [25] and deletions [12], reduced ATP generation capability [26,27], and increased susceptibility to apoptosis [28].

All these suggest OPA1 insufficiency relates to mitochondrial dysfunction, a well-known factor contributing to accelerated senescence, implicating the involvement of OPA1 in aging. Indeed, in our Drosophila model, lifespan of the dOpa1+/2 mutants was significantly reduced, demonstrating the important role of OPA1 in longevity. We also observed increased mitochondrial ROS levels and production rates in dOpa1+/2 adult Drosophila. Excessive mitochondrial ROS can shorten the lifespan by directly impairing mitochondrial genome, lipids of the inner membrane, mitochondrial protein activity and/or affecting nuclear gene transcription.

In our dOpa1+/2 Drosophila model, the most severely damaged electron transport chain unit is complex II, suggesting that complex II is an important determinant of Drosophila lifespan. This result is consistent with a comprehensive study of the lifespan in Drosophila with RNAi-inactivation of nuclear genome-encoded mitochondrial complexes I–V genes. The study shows one third of the RNAi lines that exhibited altered lifespan resulted from inactivation of complex II-encoding genes (David Walker, UCLA, personal communication).

In Hela cells, inhibition of Drp1 modifies the pattern of OPA1 isoforms, results in dysfunction of OPA1 and leads to a significant decrease in complex II respiration with complex I function intact [29]. The correlation between OPA1 defect and complex II dysfunction in both flies and Hela cells suggests that the mitochondria structure is important for integrity of complex II functions.

Mitochondrial complex II oxidizes succinate to fumarate and passes the electrons into the quinone, thus serving as the only
mitochondrial membrane complex involved in both electron transport and the tricarboxylic acid cycle. It was shown that complex II defect alone is associated with an increased level of ROS [30]. Mutation in *sdhB* in *Drosophila*, encoding for subunit B of complex II, displays structural abnormalities in the mitochondria, causes an increased level of mitochondrial hydrogen peroxide, and is associated with hypersensitive to oxygen and shortened lifespan [30]. In *Caenorhabditis elegans*, a mutation in mev-1, encoding for a subunit of complex II, also resulted in augmented ROS production and shortened lifespan [31,32]. In fact, the short-lived mutant exhibited increased level of nuclear damage, demonstrating mitochondrial derived oxidants may also be a

Figure 2. Heterozygous *dOpa1* mutation results in increased Reactive Oxygen Species (ROS) production and decreased resistance to oxidative stress. ROS production rate (A) and ROS levels (B) in *dOpa1*−/− and *dOpa1*++ Drosophila were measured by MitoSOX fluorescence. Survival curves of *dOpa1*−/− and *dOpa1*++ Drosophila exposed to 15 mM paraquat (C) indicated that *dOpa1* deficiency was associated with increased sensitivity to paraquat treatment.

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significant source of overall genomic instability [33]. Whether OPA1 deficiency leads to nuclear complex II gene transcriptional reduction, damage to complex assembly or protein function remains to be elucidated.

In contrast to our data, in OPA-RNAi MEF cells, knock down of OPA1 induced a severe reduction in respiration, and oxygen consumption for complexes I, III and IV were all compromised [34]. Fibroblast cells derived from DOA patients with missense mutation showed a significant impairment of oxidative phosphorylation (OXPHOS), mostly mediated by complex I [27]. Furthermore, Co-immunoprecipitation experiments revealed a direct interaction between OPA1 and complexes I, II, and III, but not IV [27]. The discrepancy might be associated with the type of cells investigated — cultured fibroblast cells preferentially use anaerobic glycolysis for energy production and so mitochondrial OXPHOS in MEF might be metabolically different from that in vivo. In addition, different OPA1 mutations can also result in variable energy defects [35,36]. In our experiments, mutant dOpa1 Drosophila were generated by P-element or transposon insertion and resulted in dOpa1 haploinsufficiency.

ROS can damage the electron transport chain. The respiratory chain malfunction can also lead to increased ROS production, constituting a reinforcing vicious cycle and possibly resulting in catastrophic breakdown of mitochondrial function [37]. In the dOpa1+/− flies, both increased ROS levels and compromised ETC functions were observed. It is intriguing to investigate which serves as the primary cause. To distinguish between these two possibilities, we studied mitochondrial aconitase activity. At one week of age, heterozygous mutation of dOpa1 caused a significant decrease in the complex activities, but aconitase activity was normal, suggesting that dysfunction of ETC is the primary cause. However, we also observed an age-dependent aconitase activity decline and an elevated aconitase protein level in dOpa1+/− flies after reactivation at 5 weeks of age, suggesting that the aconitase activity reduction was the consequence of inactivation of existing enzyme by increased ROS. The elevated amount of extant aconitase in dOpa1+/− flies might reflect the physiological responses to synthesize more aconitase protein to compensate for the reduction in enzymatic activities. These data indicate that decreased complex activities are the primary underlying mechanism and the secondary increase of ROS reinforces vicious cycle and results in catastrophic mitochondrial dysfunction, including decrease in ATP production. The gradual accumulation of ROS...
Figure 4. The shortened lifespan of heterozygous dOpa1 mutants is extendable through the administration of antioxidants in male flies, but not in female flies. dOpa1<sup>+/2</sup> mutants and dOpa1<sup>+/+</sup> controls were transferred to fresh food every two to three days while aging. Male and female dOpa1<sup>+/2</sup> Drosophila and dOpa1<sup>+/+</sup>Drosophila were separated and maintained on with and without 100 μM MnTBAP antioxidant food. Longevity assays were performed. MnTBAP feeding significantly extended lifespan of male dOpa1<sup>+/2</sup> Drosophila (A), but not in the females (B).

Figure 5. dOpa1<sup>+/2</sup> mitochondria are highly irregular and dysmorphic in the muscle. Tissues from adult Drosophila were analyzed by transmission electron microscopy. Mitochondrial phenotypes in muscle (A, B). The dOpa1<sup>+/2</sup> mitochondria were highly irregular and dysmorphic (Left) compared to wild type mitochondria (Right). Bar = 0.5 μm.
and resultant chronic damage to the mitochondria is consistent with the progressive manifestation of phenotypes in OPA1-related DOA patients.

Gender is an important and profound factor in aging and lifespan and it is predicted that the longer-lived gender should have lower levels of oxidative stress. In both mammals and flies, females generally have a longer lifespan, possibly due to the suboptimal mitochondrial function in the males [40]. The insulin/IGF pathway [19,41] and JNK-dependent signaling [42] are important regulators for tolerance to oxidative stress and longevity in Drosophila and both cascades can be activated by ROS. Generally, females respond better to antioxidative interventions. The lifespan extension impacts of human SOD over-expression in Drosophila were more obvious in the females in six out of ten different genetic backgrounds studied while only one male background showed significant increase [43]. Interestingly, the antioxidant rescue of lifespan for heterozygous dOpa1 mutants is only effective in the males, but not in the females in our study, which is consistent with the complex II activity. The gender-specific variation of antioxidant influence can also be explained by differences in feeding habits and/or distinct responses to intracellular redox status changes between male and female flies.

In summary, OPA1 deficiency causes malfunction of ETC and results in elevated production of ROS, which in turn can further impair the complex activity and mitochondrial bioenergetic capability and consequently accelerate aging in dOpa1 mutants. Whether OPA1 insufficiency causes ETC dysfunction and induces augmented ROS generation is independent of its role in mitochondrial dynamics and apoptosis, as suggested in the C. elegans DOA model [44], requires further elucidation. In the future, the observations from the Drosophila DOA model can be extended to human DOA subjects and have the potential to establish a new model for longevity. Data presented in this study also suggest that antioxidants can sequester ROS and may hold potential as an effective therapeutic agent in this condition.

Materials and Methods

Drosophila stocks

\[ y^{[d2]} w^{[1118]} P_{[y^{[+7.2]} = \alpha-FLPN]}^{[2]}; P_{[y^{[+7.2]} = -neoFRT]}^{[42D]} PBac(WH)CG8479 [D0777] (dOpa1\textsuperscript{-/+}) \text{ and } y^{[d2]} w^{[1118]} P_{[y^{[+7.2]} = \alpha-FLPN]}^{[2]}; P_{[y^{[+7.2]} = -neoFRT]}^{[42D]} PBac(WH)CG8479 [D0777] (dOpa1\textsuperscript{-/+}) \]

Drosophila and dOpa1\textsuperscript{+/-} stocks were used in this study. These stocks were established in a previous study [14]. dOpa1\textsuperscript{-/-} mutant type and dOpa1\textsuperscript{+/+} control were transferred to new food every two to three days while aging. dOpa1\textsuperscript{+/-} (antennae) Drosophila and dOpa1\textsuperscript{+/-} (antennae) Drosophila were heterozygous mutant or wild-type Drosophila and were kept on antioxidant food.

Longevity assay and Drosophila husbandry

Lifespan was determined under standard conditions for Drosophila husbandry (25°C, 50% Humidity, 12 hour light cycle) as described before [46]. Drosophila were collected over a 24 hour period and aged in vials containing standard Drosophila media (40–50 Drosophila/vial; same sex) and transferred every 3–6 days to fresh Drosophila media.

ROS measurements

Adult Drosophila were collected within 12 h of emergence under light CO2 anesthesia, and transferred into vials with food in groups of 15. These stocks were established in a previous study [14]. Briefly, tissue homogenates of 40 adult Drosophila were stained with MitoSOX (2.5 mM), and fluorescence was performed at 510 nm excitation/580 nm emission via a Perkin Elmer L20B luminescence spectrometer.

Paraoquat survival

Adult Drosophila were collected within 12 h of emergence under light CO2 anesthesia, and transferred into vials with food in groups of 15. Drosophila per vial, sexes separate. Seven days after emergence, Drosophila were tested for resistance to paraquat (Sigma), based on previously established methods [46]. Drosophila were starved for 2 h by placing them in empty vials, and then transferred into test vials containing filter paper disks soaked with 20 mM paraquat and 5% sucrose or 5% sucrose alone as a control. The starvation treatment ensures that initial ingestion of paraquat does not vary between lines because of differences in feeding status. Numbers of living Drosophila were recorded at 24 h intervals.

Mitochondria isolation and respiration

Mitochondria were isolated as described before [46]. Respiratory rates were determined by oxygen consumption using a Clark-type electrode and metabolic chamber containing 650 μL of reaction buffer consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl and 5 mM KH2PO4 (pH 7.2) at 25°C. Mitochondrial ATP production rates were calculated from ADP consumption rates during state III respiration.

Mitochondrial enzyme assays

Citrate synthase activity was analyzed by the reduction of 5,5′-dithiobis-2-nitrobenzoic acid at 412 nm in the presence of acetyl-CoA and oxaloacetate [47]. Aconitase activity was measured by monitoring conversion of citrate into α-ketoglutarate at 340 nm at 25°C using the coupled reduction of NADP to NADPH [48]. Aconitase was reactivated by incubation with 2 mM dithiothreitol and 0.2 mM ferrous ammonium sulfate before repeating the enzymatic activity assays.

Mitochondrial respiratory chain complex I–IV activity assay

Complex I (NADH dehydrogenase) activity was determined as the rotenone-sensitive NADH oxidation at 340 nm, using the coenzyme Q analogue 2, 3-dimethyl-5-methyl-6-n-decyl-1, 4-benzomethyluinone (DB) as an electron acceptor [47,49]. The activity of complex II (succinate dehydrogenase) was analyzed by tracking the secondary reduction of 2,6-dichlorophenolindophenol by ubiquinone-2 at 600 nm [47,49]. Complex III (cytochrome bc1 complex) activity was determined by measuring the reduction of cytochrome c at 550 nm with reduced decylubiquinone [47,49]. Complex IV (cytochrome c oxidase) activity was measured by monitoring the oxidation of reduced cytochrome c as a decrease of absorbance at 550 nm [47,49]. Complex I-IV activities were normalized by citrate synthase activity and then used in the analysis.

Transmission electron microscopy (TEM)

TEM was performed as previously described [14]. Briefly, adult Drosophila were fixed overnight in 4% paraformaldehyde at 4°C and transferred to a post-fixation solution of 1% glutaraldehyde for 1 hour, then rinsed in PBS and placed in 1% osmium tetroxide for 20–60 min. The samples were dehydrated by ethanol and propylene oxide immersion. A flat-embedding procedure was used followed by trimming of the tissue block with a single-edged razor blade under a dissecting microscope (Nikon). A short series of
ultrathin (60–30 nm) sections of the whole flies was cut from each block with an ultramicrotome (Reichert-Jung) and serial sections were collected on mesh and formvar-coated slot grids. The sections were stained with uranylacetate and lead citrate to enhance contrast. Skeletal muscle was examined with a Philips CM-10 transmission electron microscope and images of ommatidial units were captured with a Gatan digital camera.

Supporting Information

**Figure S1**

Heterozygous dOpa1 mutation causes an age-dependent gender-specific difference of complex II activity decline.

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**Author Contributions**

Conceived and designed the experiments: ST TH. Performed the experiments: ST PKL, ST. Analyzed the data: ST TH. Wrote the paper: ST DW TH.
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