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Ganoderma tsuage promotes pain sensitivity in aging mice

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Advances in modern medicine have extended human life expectancy, leading to a world with a gradually aging society. Aging refers to a natural decline in the physiological functions of a species over time, such as reduced pain sensitivity and reaction speed. Healthy-level physiological pain serves as a warning signal to the body, helping to avoid noxious stimuli. Physiological pain sensitivity gradually decreases in the elderly, increasing the risk of injury. Therefore, geriatric health care receives growing attention, potentially improving the health status and life quality of the elderly, further reducing medical burden. Health food is a geriatric healthcare choice for the elderly with *Ganoderma tsuage* (GT), a Reishi type, as the main product in the market. GT contains polysaccharides, triterpenoids, adenosine, immunoregulatory proteins, and other components, including anticancer, blood sugar regulating, antioxidation, antibacterial, antiviral, and liver and stomach damage protective agents. However, its pain perception-related effects remain elusive. This study thus aimed at addressing whether GT could prevent pain sensitivity reduction in the elderly. We used a galactose-induced animal model for aging to evaluate whether GT could maintain pain sensitivity in aging mice undergoing formalin pain test, hot water test, and tail flexes. Our results demonstrated that GT significantly improved the sensitivity and reaction speed to pain in the hot water, hot plate, and formalin tests compared with the control. Therefore, our animal study positions GT as a promising compound for pain sensitivity maintenance during aging.

Keywords Ganoderma, Antioxidant, Hypoalgesia, Aging

According to the definition of the World Health Organization (WHO), a society is considered "aging", "aged", and "super-aged" when the proportion of its elderly population aged over 65 years reaches 7%, 14%, and 20% of the total population, respectively¹. WHO statistical data points out that the ratio of the elderly population over the age of 65 years is expected to grow from 10% in 2022 to 16% in 2050². Therefore, the long-term care for the elderly is becoming increasingly important. Aging is accompanied by several chronic diseases, the most common being pain sensitivity deterioration³.

Multiple studies demonstrated that pain sensitivity reduction is a common problem in the elderly. In the experiment of David and Eugene in 1960⁴, 90 young men and women (aged 20–30 years) and 110 elderly people (aged 65–97 years) of the same community were stimulating their forehead by a heat source using a projector bulb to form a light through a lens. Gradually increasing the stimulation number, frequency, and intensity, the researchers observed eye muscle contractions and recorded the pain description of the subjects. The study demonstrated reduced pain perception sensitivity and relative response in the elderly⁴. Further studies also linked aging and pain sensitivity^{5–8}. There are also experiments comparing old and young mice, revealing that old mice also exhibit reduced pain sensitivity^{9–11}.

Reishi, a mushroom type of the *Ganoderma* genus, has been used as medicine for thousands of years in China, Korea, Japan, and other Asian regions. Its metabolites, polysaccharides, and terpenoids are functional ingredients for multiple disease prevention and treatment¹² with anticancer, blood sugar regulatory, antioxidation, antibacterial, antiviral, and liver and stomach protective properties^{13–19}. Among the different Reishi types, only red, purple, and spruce ones are officially recognized for their medical effects with *Ganoderma tsuage* (GT) being the best antioxidant^{20,21}.

Although pain is a subjective feeling that cannot be directly expressed, it could still be judged from human and animal behavioral responses, including escape, dodging, and other reactions to eliminate painful stimuli²². The pain test in mice is mainly used to evaluate analgesic effects. A standard pain test approach involves pain

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recognition by observing the licking response in mice to a hot plate^{23–25}, a tail-flick reaction by hot water^{24,26} or thermal radiation^{25,27}, and formalin assay^{24,25}. Accordingly, this study aimed at investigating whether GT would exhibit a protective effect on aging-induced pain sensitivity reduction. To the best of our knowledge, no previous study has demonstrated the correlation between GT and pain perception so far, underlining the importance of our study.

Materials and methods

Animals and treatments

We used 5-week-old male ICR mice (BioLASCO Taiwan) in this study, approved by the Institutional Animal Care and Use Committee of Chung Yuan Christian University (Approved protocol no# 105016). The animals were kept under the following conditions: 18–26 °C, 30–70% humidity, and a 12:12 h light–dark cycle with free access to laboratory chow and water throughout the study. We divided the animals into four groups as follows: Control (Control), GT treatment (GT), D-galactose (D-gal) treatment (aging), and D-gal plus GT treatment (aging + GT). In the D-gal treatment group, we subcutaneously administered daily D-gal injections (100 mg/kg)²⁸ to the mice for eight weeks. In the D-gal plus GT treatment group, GT (200 µg/kg)²⁸ was administered as oral gavage daily concomitantly with D-gal injections for eight weeks. All control animals were subcutaneously administered phosphate-buffered saline at equal volumes. In the GT treatment group, oral gavage was performed daily for eight weeks. At the end of the final week, we performed the behavior tests with the mice, then sacrificed them and conducted biochemical analyses. The behavior tests are described in detail below. The mice were sacrificed with carbon dioxide inhalation in the original feeding box, which was placed in the euthanasia container. Carbon dioxide was gradually filled the euthanasia container at a rate of 20–30% of the chamber volume per minute, causing the animal to lose consciousness quickly. Carbon dioxide was continuously perfused for another five minutes after the animal appeared dead. If mice showed anxiety during the sacrifice, 4–5% isoflurane was given to induce an anesthetic effect that caused them to lose consciousness before the carbon dioxide perfusion. Mice's death was confirmed before removing it from the euthanasia container. All methods were carried out in accordance with relevant guidelines and regulations of NIH Guide for the Care and Use of Laboratory Animals, and all methods are reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines²⁹.

Ganoderma tsugae extraction

GT samples were gifts from Professor Ruey-Shyang Hseu, Department of Biochemical Science and Technology, National Taiwan University. Dr. Hseu entrusted the Li-Kang Biotechnical Co., Ltd (I-Lan, Taiwan) to culture and collect the mycelium. He further extracted the GT from the fruiting body using hot water as described previously³⁰. Briefly, the fruiting body was homogenized in sterilized water. The collected sample was frozen at –20 °C, vacuum-dried for 36 h, then stored at –20 °C until further use. Dr. Hseu provided and authorized us to use the water extract for this study. The crude mixture contained 1.96% triterpenes and 3.93% polysaccharides³¹. The dried extract was further analyzed for the total water-soluble polysaccharide, total triterpenoid, beta-D-glucan, and heavy metal contents. For total water-soluble polysaccharides, the sample was extracted, the polysaccharides were precipitated with ethanol, re-dissolved, reacted with phenol–sulfuric acid, and analyzed under a UV–Vis Spectrophotometer (UV–Vis) (ChromTech® CT-2200, Taiwan). The amount of total water-soluble polysaccharides was 45.4 g/100 g. For total triterpenoids, the sample was extracted, reacted with a vanillin–glacial acetic acid–perchloric acid solution as a color-developing agent, and analyzed under a UV–Vis. We registered the amount of total triterpenoids as 463 mg/100 g. Concerning beta-D-glucans, the sample was dissolved to remove low-molecular-weight sugar, hydrolyzed by enzyme specificity, and analyzed using a High-Performance Anion Exchange Chromatography–Pulsed Amperometric Detector (HPAEC–PAD) (Thermo Fischer Scientific, Germering, Germany). The amount of beta-D-glucans was 0.24 g/100 g. Finally, we detected no heavy metals. The GT water extraction was followed by DMSO precipitation, reserve dialysis, and protein depletion. The final triterpenoid and water-soluble polysaccharide content was 128 and 0.39 g/100 g in GT–DMSO solution, respectively. Our previous preliminary study followed a GT dosage of 200 µg/kg/day³².

Open-field test (OPT)

Open-field test was performed based on the previous publication with modification³³. An acrylic box measuring approximately 100 × 100 × 30 cm was divided by gridlines into nine equally sized squares that served as nine zones. Each mouse was placed individually in the central square. Crossing was defined as exiting from the previous square with all four paws. Between the tests, the acrylic box was wiped with 75% alcohol to avoid odor interference. Crossing frequency and duration spent in the center square within five minutes was recorded for each mouse. Behavioral data were acquired by video recording and software analysis (Noldus Information Tech, Ethovision XT 10.0, Wageningen).

Tail-flick test-hot water

The tail-flick test-hot water, in general pain-related experiments, is mostly a one-time test for analgesic evaluations^{24,26}. Briefly, mice were put in the restrainer, exposed, and soaked the rear 2/3 tail in a constant temperature water bath at 55 °C. The time was recorded from tail soaking to flicking. In order to avoid scalding the tail, the soaking time did not exceed 15 s. If no tail flicking occurred in 15 s, we stopped scalding and removed the mouse.

Tail-flick test-hot plate

The hot plate test is performed based on the previous report with modifications³⁴. The thermal stimulation was performed using the hot plate (Panlab model LE7406, Harvard Apparatus, Holliston, MA). First, we set the hot plate tester to 55 °C, and when the temperature reached 55 °C, we put the mouse into the acrylic cover to measure the time it licked its feet. If no reaction was given in 90 s, we removed the mouse from the hot plate to prevent tissue damage and recorded the time as 90 s.

Formalin pain test

The formalin pain test follows the process^{35,36}: The mice were placed in a transparent 9.5 cm-diameter and 10 cm-tall jar for 5 min to adapt to the environment, followed by a 20 µL 2% formalin injection into the right hind paw. We recorded the number of mice licking their feet between 0 and 10 min after the injection (pain stimulation) and the time between 10 and 30 min (inflammatory response of the surrounding tissue).

Statistical analysis

The data are represented as the mean ± standard error. The statistical significance ($p < 0.05$) was determined using the GraphPad InStat software 3.05 package for Windows (GraphPad Software Inc., San Diego, CA).

Results

No significant change in the physiological value of the mice in different groups

First, we measured and recorded the body weight and basic hematology values of the mice, detecting a healthy weight increase each week with no significant difference between the GT-treated and control animals (Fig. 1). Moreover, the hematology results in mice demonstrated that the white blood cell (WBC), red blood cell, and hemoglobin values fell in the healthy range in all groups with no significant differences, although aging mice displayed higher WBC numbers (Table 1).

D-gal and GT have no significant effect in open-field test

The OPT evaluates alertness in mice. In general, mice are more alert to new environments, they thus tend to move along corners and walls and are less likely to walk into open fields (e.g., the middle of a box). Our data revealed that mice in each group stayed less in the central quadrant (Fig. 2A). The mobility of mice could be judged by their movement frequency in each quadrant and accumulative time in the field (Fig. 2B,C). Compared with the control, the movement frequency of the aging group displayed a downward trend, near the level of significance (Fig. 2B). In contrast, the GT group was more active than the aging group, although no significant difference could be observed among the groups. Finally, no significant difference could be detected between the groups for a cumulative duration either (Fig. 2C).

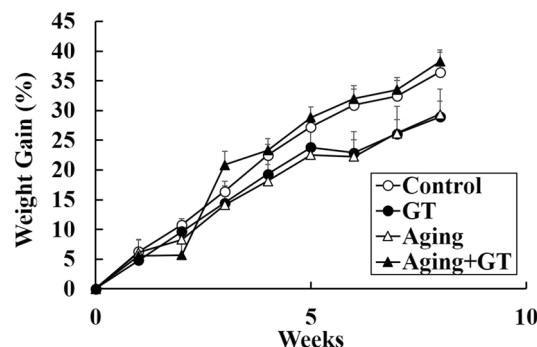


Figure 1. Body weight changes in mice during the experiment. The body weight increased healthily in each group with no significant differences. $N = 6$ except control ($N = 5$).

Groups	Parameters*			
	Control	GTDE	D-gal	D-gal + GTDE
Hemoglobin (g/dL)	12.76 ± 0.59	11.30 ± 0.50	12.97 ± 1.07	13.67 ± 0.93
RBC (10^9 /mL)	5.38 ± 0.42	5.69 ± 0.95	6.24 ± 1.64	6.37 ± 2.03
WBC (10^6 /mL)	4.01 ± 0.27	4.15 ± 0.41	5.72 ± 0.91	4.49 ± 0.68

Table 1. Changes in physiological parameters of ICR. *The differences in hemoglobin level, RBC count, and WBC count were not statistically significant. The physiological parameters were compared using the one-way ANOVA followed by the Tukey–Kramer multiple comparisons test. Means ± SEM, $n = 6$.

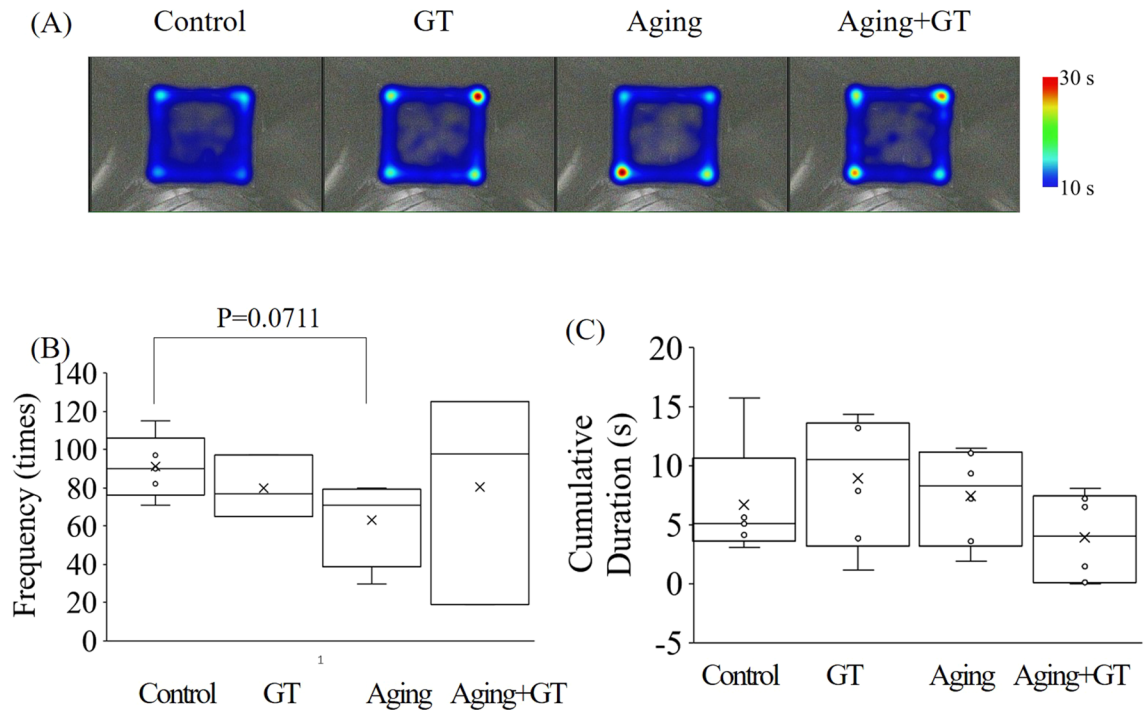


Figure 2. Open-field test, indicating no difference in locomotion between the groups. (A) Heat map diagrams between the groups. Warm and cold colors refer to longer and shorter stay in the area, respectively. (B) Frequency quantification of passing the center in each group. The data indicates a lower frequency in the aging group compared to the control with a nearly significant difference. (C) Cumulative duration of staying in the center area with no differences among the groups. N=6 except control (N=5).

GT gavage improves the reaction time in the Tail-flick test against hot water stimulation

We applied the hot water pain test to assess the response time of mice to painful stimulations. We subtracted the data of week 0 from that of each week to reveal a potential increase or decrease in the reaction times relative to week 0 before D-gal induction and GT gavage. Negative and positive values indicated shorter and longer reaction times (i.e., faster and slower reactions) in a given week compared to week 0, respectively. Overall, the reaction time in the control group slightly reduced and that in the GT group reduced even more, indicating shorter reaction times required for the tail-flick reaction in mice compared to week 0 (Fig. 3). Moreover, we observed no significant difference between these two groups. However, the reaction time in the aging group significantly increased, which could be significantly reduced by GT, bringing it closer to the standard reaction time.

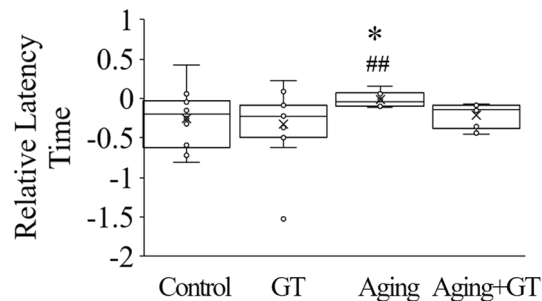


Figure 3. GT gavage prevents slow reaction times in D-gal-induced aging mice during the tail-flick pain sensitivity test upon hot water stimulation. The results confirmed that the GT-fed and aging groups displayed a faster and slower reaction speed, respectively. * $p < 0.05$ compared to the control and ## $p < 0.01$ compared to the GT group by Student's t-test. $n = 2$ (2 tests/mice), N=6 except control (N=5).

GT prevents the D-gal-induced increase in reaction time in the tail-flick test by hot plate stimulation test

The paw-licking response in mice was tested using a hot plate apparatus. Our results demonstrated that the reaction time of the aging group was significantly longer compared to the control (Fig. 4). Upon GT administration, the reaction time returned to the level of that in the control, displaying no significant difference.

GT prevents the D-gal-induced pain reaction in the formalin test

The foot licking response in mice upon formalin injection could be divided into two intervals for analysis and judgment. In phases I (0–10 min) and II (10–30 min), the number of paw-licking events in the D-Gal treatment group increased and decreased compared with the control, respectively (Fig. 5). Moreover, both in phase I and phase II, the number of paw licks significantly increased in the GT group. Besides, in phase I, GT treatment has no significant effect on the paw licking between the aging + GT group and the aging group. However, in phase II, paw-licking events significantly increased and recovered to the control level.

Discussion

The weight of the mice increased healthily during the experiment without any excessive stress or sudden weight loss due to the experimental operation, drug administration, and induced aging (Fig. 1). The field results revealed two main behaviors: alertness and activity. Since mice exhibit better alertness to their environment in general, they would avoid exploring unknown areas (the central quadrant) and move mostly along the walls^{37,38}. Induced aging and GT administration did not change the alertness in mice (Fig. 2A,C). However, moving frequency between each quadrant, which could be considered as an locomotor activity indicator in mice, decreased in mice of the aging group (Fig. 2A,C). Previous studies underlined that aging might lead to reduced locomotor activity in mice^{7,39}, which is in good agreement with our experimental results and hints that our induction of aging was successful. Among the experimental groups that with induced aging and GT administration exhibited better

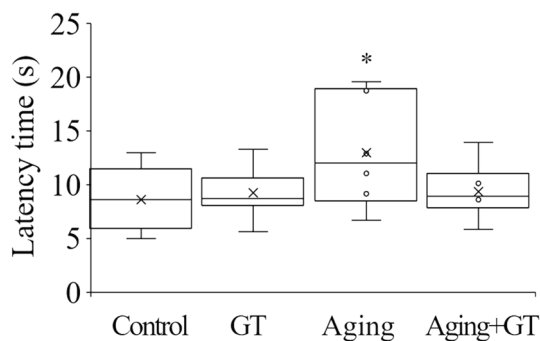


Figure 4. D-gal-induced aging reduced traction in the tail-flick test for pain sensitivity upon hot plate stimulation. Compared with the control, reaction time of the aging group significantly increased, and it recovered upon GT administration. * $p < 0.05$ compared to the control using Student's t-test. N=6 except control (N=5).

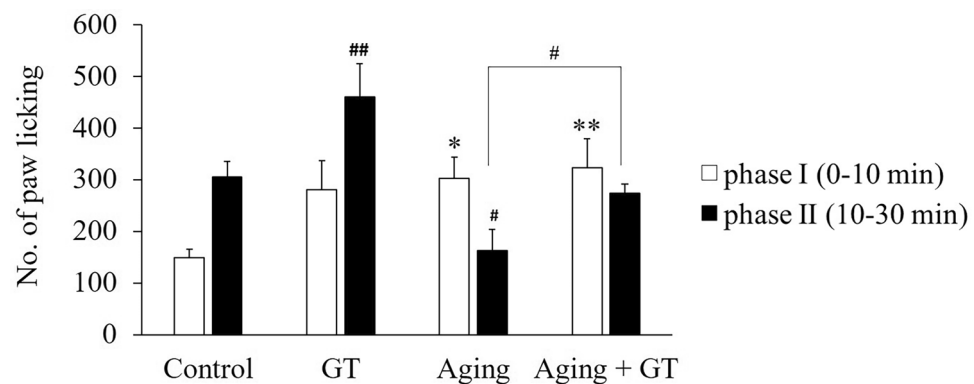


Figure 5. Formalin test to assess pain reaction in the right paw of mice upon D-gal-induced aging. In phase I (0–10min), the number of mice licking the paw increased in the D-Gal aging group compared to the control. In phase II (10–30min), the number of paw licking in the GT group significantly increased compared with the control. The number of paw-licking events in the aging group significantly decreased, which could be reversed by GT. * $p < 0.05$, ** $p < 0.01$ compared to the control of phase I using Student's t-test and # $p < 0.05$, ## $p < 0.01$ compared to the control of phase II by Student's t-test. N=6 except control (N=5).

mobility than the induced aging-only group. Therefore, we could imply that galactose administration could indeed trigger aging in mice and reduce their mobility^{40,41}, and GT could prevent this phenomenon.

Concerning the reaction time in hot water, while the aging group required longer reaction time than the control⁴², GT administration improved pain sensitivity in mice, thereby reducing the reaction time to the control level (Fig. 3). Furthermore, the hot plate-related results for paw-licking reaction testing demonstrated that aging mice needed a longer reaction time following hot plate stimulation, thereby confirming that pain sensitivity is lower in aging mice^{43,44}. This phenomenon could be prevented by GT administration, indicating that GT could contribute to maintaining a healthy pain sensitivity system.

The response to the formalin test could be divided into two main periods and analyzed separately. Higher numbers of paw-licking events indicate higher-level pain sensation in mice. In phase I (0–10 min), the pain response is caused by a foreign matter invading the sole, the number of paw licks thus increased in the GT, aging, and aging + GT treatment groups. In phase II (10–30 min), the pain is caused by inflammation^{35,45–47}, leading to significantly higher numbers in paw-licking events in the GT treatment group compared to the control (Fig. 5). The d-gal aging groups were significantly lower than the control group, but the number of paw licks in the aging + GT group has recovered. Based on phase II, GT administration could increase pain sensitivity in mice. Aging reportedly reduces pain sensitivity⁴⁸. Human studies also showed that pain sensitivity is decreased with aging^{3,8}, while other groups show opposite results⁴⁶. Moreover, data have shown that elderly subjects appear to be more susceptible to long-term pain, and drugs that target peripheral sensitization are less effective⁴⁶. Therefore, according to our animal study, GT could be a potent treatment to overcome aging-induced pain sensitivity reduction.

The limitation of our study is that we only report GT's effect on males. Literature, both in humans and rodents, shows that females have a lower pain threshold. Therefore, the pain sensitivity in females is higher than in males^{49–53}. One researcher has the opposite opinion: From the point of view of the dentist, women have a higher tolerance to pain than men⁵⁴. However, the literature illustrated that pain sensitivity depends on the type of pain source: Some show female sensitivity higher than males^{49–53}, while others show no differences^{55,56}. Even more, some reports show that female has a different tolerance to pain in the hormone cycle^{57–59}. Moreover, for the same reason, males show higher reactivity and effect than females to the pain-killing drug or related chemicals treatment^{53,60}. Thus, males have a more stable reaction to the pain sensation and therapy. We chose male mice for our model first. Accordingly, in the future direction of our study, we will not only look at the GT effect against pain in both genders, but we might also examine it in different degrees of aging.

Conclusion

This is the first study applying Reishi in reduction of pain sensitivity during aging. The results of this animal study demonstrated that aging reduces pain sensitivity, which could be potentially restored by GT in mice, thereby providing a potential candidate to solve the problem of reduced pain sensitivity against aging.

Data availability

Data is provided within the manuscript or Supplementary Information files.

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K.Y., C.L., and C.T. wrote the main manuscript text. C.L., W.L., and C.T. performed the experiments. C.L., W.L., C.T., and J.P. prepared figures. C. T. designed and investigated the project. All authors reviewed the manuscript.

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Competing interests

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