Autophagy-related gene 7 is downstream of heat shock protein 27 in the regulation of eye morphology, polyglutamine toxicity, and lifespan in *Drosophila*

Shih-Fen Chen,† Ming-Lun Kang,† Yi-Chun Chen, Hong-Wen Tang, Cheng-Wen Huang, Wan-Hua Li, Chun-Pu Lin, Chao-Yung Wang, Pei-Yu Wang, Guang-Chao Chen, and Horng-Dar Wang

Abstract

**Background:** Autophagy and molecular chaperones both regulate protein homeostasis and maintain important physiological functions. Atg7 (*autophagy-related gene 7*) and Hsp27 (*heat shock protein 27*) are involved in the regulation of neurodegeneration and aging. However, the genetic connection between Atg7 and Hsp27 is not known.

**Methods:** The appearances of the fly eyes from the different genetic interactions with or without polyglutamine toxicity were examined by light microscopy and scanning electronic microscopy. Immunofluorescence was used to check the effect of Atg7 and Hsp27 knockdown on the formation of autophagosomes. The lifespan of altered expression of Hsp27 or Atg7 and that of the combination of the two different gene expression were measured.

**Results:** We used the *Drosophila* eye as a model system to examine the epistatic relationship between Hsp27 and Atg7. We found that both genes are involved in normal eye development, and that overexpression of Atg7 could eliminate the need for Hsp27 but Hsp27 could not rescue Atg7 deficient phenotypes. Using a polyglutamine toxicity assay (41Q) to model neurodegeneration, we showed that both Atg7 and Hsp27 can suppress weak, toxic effect by 41Q, and that overexpression of Atg7 improves the worsened mosaic eyes by the knockdown of Hsp27 under 41Q. We also showed that overexpression of Atg7 extends lifespan and the knockdown of Atg7 or Hsp27 by RNAi reduces lifespan. RNAi-knockdown of Atg7 expression can block the extended lifespan phenotype by Hsp27 overexpression, and overexpression of Atg7 can extend lifespan even under Hsp27 knockdown by RNAi.

**Conclusions:** We propose that Atg7 acts downstream of Hsp27 in the regulation of eye morphology, polyglutamine toxicity, and lifespan in *Drosophila*.

**Keywords:** Atg7, Hsp27, Neurodegeneration, Lifespan, *Drosophila*

Background

The aging process results from imbalanced homeostasis combined with accumulating macromolecular damage due to different intrinsic and environmental stresses [1-3]. Protein homeostasis is important in maintaining physiological function to protect against cellular degeneration [4]. Autophagy and molecular chaperones are two defensive systems utilized to uphold cellular protein quality and homeostasis [5, 6].

Macroautophagy (herein called autophagy) is a cellular, catabolic process that breaks down and recycles macromolecules and organelles under starvation conditions. Autophagy function is executed by a series of autophagy related genes (*Atg*) which are evolutionarily conserved from yeast to mammals [7]. Autophagy participates in many physiological functions including aging and neurodegeneration [8, 9], and mounting evidence demonstrates that autophagy participates in the regulation of
lifespan in different species [10-12]. In *C. elegans*, loss-of-function of *bec-1*/*Atg6* or RNA interference-mediated depletion of *Atg-7* or *Atg-12* inhibits the extended lifespan in *daf-2* mutants [13, 14], and the knockdown of *bec-1* or *Atg7* by RNAi abolishes dietary restriction-mediated longevity in *eat-2* mutants [15]. In addition, mutations in *Atg1*, *Atg7*, *Atg18*, and *bec-1* reduce lifespan in *C. elegans* [16]. In *Drosophila*, *Atg7*-null mutants are short-lived and hypersensitive to starvation and oxidative stress [17], and the neuronal overexpression of *Atg8a* regulates lifespan and tolerance to oxidative stress [18]. *Atg7* is an E1-like enzyme and is important for the membrane elongation of the autophagosome [7]. *Atg7* deficient mice exhibit polyubiquitinated protein accumulation and neurodegeneration [19] and higher levels of polyubiquitinated proteins have been detected in the aging *Atg7* mutant fly head [17]. Autophagy also protects against neurodegeneration [20] and the induction of autophagy by the reduction of TOR (target of rapamycin) activity reduces polyglutamine toxicity in both fly and mouse [21]. Suppression of basal autophagy in the central nervous system causes neurodegeneration in *Atg7* conditional knockout mice [19, 22].

Molecular chaperones modulate protein re-folding and facilitate the degradation of denatured proteins. Molecular chaperones are also implicated in several physiological functions: autophagy, neurodegeneration, stress tolerance, and aging [23-25]. Heat shock protein 27 (Hsp27) is a member of the ATP-independent, small heat shock protein family. Hsp27 null mutants exhibit decreased lifespan and reduced starvation tolerance [26], while the overexpression of Hsp27 increases lifespan and enhances stress resistance in *Drosophila* [27, 28]. Overexpression of Hsp27 prevents cellular polyglutamine toxicity and rescues the mosaic eyes induced by mild polyglutamine toxicity [27, 29].

Both Hsp27 and Atg7 are involved in maintaining protein quality and modulating lifespan and neurodegeneration. However, the interaction between Hsp27 and Atg7 is unknown. We report here that Atg7 is downstream of Hsp27 in the regulation of eye morphology, polyglutamine toxicity, and lifespan in *Drosophila*. The levels of Hsp27 and Atg7 both regulate eye morphology and the polyglutamine toxicity of 41Q. The overexpression of Atg7 rescues both the rough eye phenotype resulting from knockdown of Hsp27 as well as the more severe mosaic eye phenotype induced by the knockdown of Hsp27 under 41Q toxicity. In addition, the expression of Atg7 regulates lifespan in *Drosophila* and the enhanced lifespan seen with the overexpression of Hsp27 requires the expression of Atg7. Together we provide several lines of genetic evidence linking Hsp27 to Atg7 in the modulation of eye morphology, polyglutamine toxicity, and lifespan regulation.

### Methods

#### Fly strains and maintenance

The RNAi lines were obtained from Vienna *Drosophila* RNAi Center (VDRC), *UAS-hsp22* RNAi (#43632), *UAS-atg12* RNAi (#107317), *UAS-atg5* RNAi (#104461), *UAS-atg3* RNAi (#45560), *UAS-atg8a* RNAi (#43096), *UAS-atg8b* RNAi (#43097), *UAS-atg18* RNAi (#10045), *UAS-atg12* RNAi (#102362), *UAS-atg4* RNAi (#105366), GMRL-Gal4; UAS-41Q and GMRL-Gal4/Cyo; UAS-hsp27 were provided by Dr. Parsa Kazemi-Esfarjani. To generate *UAS-Atg7* transgenic flies, the EST clone RE27292 containing the full-length *Atg7* was used to amplify the coding sequence by the primers (forward: 5'-GTACTCGAGAAGCAAAACATGAGCAGG-3' and reverse: 5'-CATAGATCTATCCTCGTCTGGATCGGA-3') and subcloned into the Xhol and BglII sites of the transgenic vector, pIndY6 [28]. The resultant construct was verified by DNA sequencing to confirm that no mutations derived from PCR amplification were made, and injected into *w^{118}1* eggs for the generation of *UAS-Atg7* transgenic flies. All flies were maintained on standard fly food as described in Liu et al. [30] and incubated at 25°C, 65% humidity, in a 12 h/12 h light/dark-cycle fly incubator.

#### Fly eye image

Two-day-old flies of the different types were anaesthetized by carbon dioxide on a porous platform and the eye images were taken by light microscopy (SMZ1500, Nikon). For the scanning electron micrograph, the fly was fixed on a copper stage and the fly eye image was acquired by scanning electron microscopy (TM-1000, Hitachi). For each fly line, a total of more than 86 eye images from at least three independent crosses were examined.

#### RT-PCR and real-time PCR

Total RNA was prepared from about 20 flies of each specific allele and homogenized in 1 ml Trizol solution. Equal amounts (1 μg) of each DNase I-treated RNA were reverse-transcribed to cDNA with MMLV reverse transcriptase (Promega). The cDNAs were used as templates for RT-PCR or real-time PCR as described in Liu et al. [30]. The information of the primers is available upon request.

#### Lifespan and starvation assays

For the lifespan assay, all the fly lines have been outcrossed with *w^{118}1* as described previously [31]. The newly eclosed flies of each allele were collected by sex with 30 flies per vial, maintained at 25°C, 65% humidity in a 12 h/12 h light/dark-cycle fly incubator and transferred to a new vial every 3 or 4 days until all were dead. The statistical significance was calculated by log rank test. At least three independent measurements were performed for each experiment.
For the starvation assay, newly eclosed flies of each type were collected by sex with 20 flies per vial and recovered overnight. Next day the flies were transferred to the vials with 1% agar and transferred to new agar vials daily. The numbers of the dead flies were recorded every 4 hours until all were dead. The statistical significance was calculated by student’s t test.

**Immunofluorescence**

GFP-NLS-marked Atg7 or Hsp27 RNAi knockdown clones in the larval fat body were generated by heat shock-independent FLP/FRT induction as described previously [32, 33]. FLP/FRT method allows to examine the mitotic GFP-NLS-marked RNAi knockdown clones surrounded by the control cells that do not incorporate the RNAi knockdown in the same tissue under the same condition [33]. Fat bodies from early third instar larva cultured in standard fly food with yeast paste (fed condition) or in dishes containing 20% sucrose only (starvation condition) for 4 hr were dissected and fixed with 4% paraformaldehyde and then examined by confocal laser scanning microscope (LSM510; Carl Zeiss Inc.) equipped with a 63x Plan-Apochromat (NA1.4) objective lens.

**Results**

**Autophagy-related gene 7 is downstream of heat shock protein 27 in the regulation of Drosophila eye phenotype**

Protein homeostasis plays an important role in lifespan and stress response [1, 2]. Heat shock protein 27 (Hsp27) has been shown to regulate lifespan and response to different stresses [26-28]. Autophagy-related gene 7 (Atg7) is required for normal lifespan and tolerance to starvation and oxidation [17]. However, the genetic interaction between Hsp27 and Atg7 is unknown. We examined the effects of altering Hsp27 and Atg7 expression in the Drosophila eye using the GMR-Gal4 driver followed by the analyses of eye morphology utilizing scanning electron microscopy and light microscopy. Overexpression of Hsp27 or Atg7 results in a normal eye phenotype and regular ommatidia shape as seen in the GMR-Gal4 control flies (Figure 1, A-A’, B-B’, D-D’). Interestingly, knockdown expression of either Hsp27 or Atg7 by expression of interfering RNAs using GMR-Gal4 results in similar rough eye phenotypes with fused and enlarged ommatidia (Figure 1, C-C’, E-E’). Overexpression of Atg7 in the Hsp27 knockdown background fully rescues the rough eye phenotype of the Hsp27 knockdown (Figure 1, F-F’). However, overexpression of Hsp27 in the Atg7 knockdown background fails to rescue the rough eye phenotype of the Atg7 knockdown (Figure 1, G-G’). These results suggest that Atg7 is located downstream of Hsp27 in the regulation of Drosophila eye morphology. To further confirm that Hsp27 and Atg7 function in the same pathway controlling eye phenotype, we examined whether there is any additive effect on fly eye morphology by either the co-overexpression or co-knockdown of Hsp27 and Atg7. The overexpression of both Hsp27 and Atg7 in combination produces a normal eye phenotype, similar to the overexpression of Hsp27 or Atg7 alone (Figure 1, B-B’, D-D’, H-H’). The simultaneous knockdown of Hsp27 and Atg7 does not further deteriorate the rough eye phenotype when compared to the effects of either gene alone (Figure 1, C-C’, E-E’, I-I’), implying that Hsp27 and Atg7 operate in the same pathway. These data provide the first evidence that Atg7 is downstream of Hsp27 in the regulation of Drosophila eye morphology.

**Knockdown of other autophagy-related genes and heat shock protein 22 does not result in a rough eye phenotype in Drosophila**

To determine whether the rough eye phenotype is specific to Atg7, or whether it represents a general effect of altering autophagy, the effects of knockdown of additional autophagy-related genes were examined by using GMR-Gal4 and none of these displayed the rough eye phenotype (Figure 2, Figure 1, E-E’). Knockdown of Atg1 shows a normal eye phenotype (Figure 2, A-A’, Figure 1, A-A’), while knockdown of other autophagy-related genes: Atg 4, 5, 8, 9, 12, 18 displayed subtle eye color phenotypes but had no effect on the ommatidia structure (Figure 2, B-G, B’-G’; B”-G”). These data suggest that the rough eye phenotype resulting from Atg7 knockdown is Atg7-specific and not involved in the alteration of other autophagy-related genes. Similarly, to examine whether the rough eye phenotype is specific to Hsp27 knockdown, we tested the effects of knockdown of Hsp22, another known lifespan modulation gene [34], by GMR-Gal4 and did not observe any effects on the eye like that of Hsp27 knockdown (Figure 1, C-C’). Q-PCR analysis confirms that there is reduced expression of Atg and Hsp22 genes in the RNAi knockdown experiments (data not shown). Thus the rough eye phenotype is specific to the knockdown of either Atg7 or Hsp27.

**Knockdown of Atg7 but not Hsp27 blocks starvation-induced autophagosome formation**

To verify that the knockdown of Atg7 by UAS-Atg7RNAi from VDRC can affect starvation-induced autophagy, we generated UAS-Atg7RNAi clones in the fat-body by using the FLP/FRT method [32, 33] and examined the distribution of mcherry-Atg8a puncta. The distribution of mcherry-Atg8a is in a uniformly diffuse structure under optimal feeding conditions (Figure 3, B, J) and becomes localized to punctate structure under starvation conditions (Figure 3, F, N). Under starvation conditions, the GFP-NLS clones with the Atg7 knockdown, where the cells are circled by dotted line, display a reduced number of mcherry-Atg8a puncta than the surrounding control clones without Atg7 knockdown.
knockdown which have no GFP-NLS signal (Figure 3, E, F). These results demonstrate that knockdown of Atg7 by UAS-Atg7 RNAi is able to block mcherry-Atg8a mediated autophagosome formation under starvation. To examine whether knockdown of Hsp27 can alter autophagosome formation, we also generated UAS-Hsp27 RNAi clones in the fat body and inspected the distribution of mcherry-Atg8a puncta. Under starvation, the autophagosome formation...
indicated by mcherry-Atg8a puncta is not altered by comparing the GFP-NLS marked Hsp27 RNAi knockdown clones, which are circled by dotted line, to the surrounding control clones without GFP-NLS signal and no Hsp27 RNAi knockdown (Figure 3, M, N). The data indicate that Hsp27 knockdown does not block the mcherry-Atg8a mediated autophagosome formation under starvation. The notion is consistent with the previous data since knockdown of Atg8 does not result in the rough eye as the knockdown of Hsp27, suggesting that Hsp27 and Atg8 do not function in the same genetic pathway.

**Atg7 and Hsp27 attenuate the mild polyglutamine toxicity of 41Q but cannot rescue longer polyglutamine tract toxicity by 63Q**

Overexpression of Hsp27 can rescue the mosaic eye phenotype resulting from mild polyglutamine (41Q)-induced toxicity but not the rough eye phenotype resulting from severe polyglutamine (127Q) toxicity [27]. Since Atg7 acts downstream of Hsp27 in the eye, we were interested in determining whether the overexpression of Atg7 would also only rescue mild polyglutamine phenotypes. As with Hsp27, the overexpression of Atg7 rescues the mosaic eye phenotype caused by 41Q (Figure 4, A, B, D) but cannot rescue the more severe, rough eye phenotypes resulting from the longer polyglutamine tract of 63Q (Figure 4, G, H, J). The knockdown of either Hsp27 or Atg7 enhances the pigmentation phenotype observed in the eye of flies expressing 41Q. Interestingly only the knockdown of Atg7, but not that of Hsp27, enhances the eye morphology phenotype (rough eye) in combination with 41Q overexpression (Figure 4, C, E). The knockdown of Hsp27 or Atg7 does not exacerbate the rough eye phenotypes of 63Q (Figure 4, I). Interestingly, the overexpression of

![Figure 3 Starvation-induced Autophagosome Formation is Inhibited by RNAi-mediated Depletion of Atg7 but not Hsp27.](http://www.jbiomedsci.com/content/19/1/52)
Atg7 partially rescues the more dramatic mosaic eye phenotype induced by Hsp27 knockdown in the 41Q background (Figure 4, C, F), supporting the idea that Atg7 is downstream of Hsp27 in the alleviation of 41Q toxicity. However, the combination of the overexpression of Atg7 and knockdown of Hsp27 do not change the rough eye phenotype of 63Q (Figure 4, L).

**Atg7 regulates lifespan and is required for Hsp27-mediated extended lifespan in Drosophila**

Hsp27 levels are likely to regulate Drosophila lifespan since Hsp27 overexpression extends Drosophila lifespan [27, 28] while the knockout Hsp27 mutant is short-lived [26]. The knockdown of Hsp27 by either hs-Gal4, or da-Gal4 exhibits reduced Hsp27 levels and displays a 20% (P < 0.001), and 27% (P < 0.001) decrease in mean lifespan, respectively (Figure 5, A - D; Additional file 1: Table S1). Since Atg7 is downstream of Hsp27 in the regulation of eye morphology and mild polyglutamine toxicity, and Atg7 null mutants display shortened lifespan [17], we tested whether Hsp27-mediated enhanced lifespan requires Atg7. Atg7 overexpression by hs-Gal4 shows a robust increase in Atg7 transcripts relative to control flies and increases the mean lifespan by about 11% (P < 0.01) relative to the control flies (Figure 5, E and G; Additional file 2: Table S2). Conversely, knockdown of Atg7 by hs-Gal4 exhibits reduced levels of Atg7 transcripts and decreases mean lifespan by about 10% (P < 0.01) when compared to the control flies (Figures 5F and H; Additional file 2: Table S2). These results indicate that like Hsp27, Atg7 levels also regulate Drosophila lifespan.

It has been shown that neuronal overexpression of Atg8a by appl-Gal4 extends Drosophila lifespan and increases resistance to starvation [18]. To test whether neuronal overexpression of Atg7 enhances lifespan and starvation resistance, Atg7 was overexpressed in neurons using appl-Gal4, resulting in increases of 12% (P < 0.001) in mean lifespan and 18% (P < 0.01) in starvation resistance (Figure 5, I; Additional file 2: Table S2 and Additional file 3: Table S4). In addition, the simultaneous overexpression of Atg7 and knockdown of Hsp27 results in flies that exhibit a 21% (P < 0.001) extension in mean lifespan (Figure 5, J). Conversely, the flies possessing both knockdown of Atg7 and overexpression Hsp27 display a reduction of 27% (P < 0.001) in mean lifespan relative to the control flies (Figure 5, J; Additional file 4: Table S3). To further demonstrate that Atg7 functions downstream of Hsp27, we carried out the locomotion assay to measure the climbing activity of the flies with the different combination of overexpression and knockdown of Atg7 and Hsp27 along with the control flies under paraquat-induced oxidative stress. Similar to the lifespan result, the flies with simultaneous overexpression of Atg7 and knockdown of Hsp27 displayed significantly better climbing activity (42%, P < 0.001) than that of the control flies (22%), and the flies with simultaneous knockdown of Atg7 and overexpression of Hsp27 exhibited a significantly lowered locomotion activity (15%, P ≤ 0.01) than that of the control flies.

![Figure 4](https://example.com/image.jpg)
Figure 5 (See legend on next page.)
specific to these particular molecules since the knockdown of heat shock proteins may rely upon autophagy to reduce polyglutamine toxicity. For example, the anti-polyglutamine-aggregation activity of Hsp70, one of the human small heat shock proteins, was substantially diminished in Atg5-deficient cells [40]. In addition, it is possible that the small heat shock protein Hspb8-Bag3 complex enhances Htt43Q degradation via autophagy since the treatment of the Htt43Q transfected HEK-293T and COS1 cells with an autophagy inhibitor significantly reduced Hspb8-Bag3-mediated Htt43Q degradation [41]. Furthermore, it was recently suggested that the small heat shock protein HspB7 assists in the loading of misfolded proteins or aggregates in autophagosomes [42]. Together, these findings indicate that autophagy is downstream of small heat shock proteins and support our results that Atg7 is downstream of Hsp27.

The inhibition of autophagy results in decreased lifespan. Atg7 activity is essential for the longevity resulting from either reduced insulin signaling or caloric restriction in which depletion of Atg7 was found to block the longevity phenotypes of both dafl-2 and eat-2 mutants [13, 15]. Our data showed that RNAi knockdown of Atg7 by hs-Gal4, starting from embryonic to adulthood stage, results in a shortened lifespan similar to that of the Drosophila Atg7 null mutant [17]. Loss-of-function mutations in Atg7 as well as Atg1, Atg18, and Beclin-1 shorten lifespan in C. elegans [16]. Several autophagy mutants including Atg7 were identified chronologically short-lived in a yeast genetic screen [43]. However, it should be noted that not all autophagy genes are linked to aging and Atg7 is one of the conserved Atg genes that is involved in the regulation of aging in most species [9]. Conversely, the induction of autophagy increases lifespan. The induction of autophagy by caloric restriction or reducing target of rapamycin (TOR) activity enhances lifespan [9] and the neuronal overexpression of Atg8a increases Drosophila lifespan [18]. We have found that the overexpression of Atg7 extends lifespan in Drosophila and that the neuronal overexpression of Atg7 is sufficient to reverse Hsp27-knockdown-mediated, shortened lifespan. Knockdown of Atg7 blocks Hsp27-mediated extended lifespan, again supporting the model that Atg7 acts downstream of Hsp27 in the regulation of lifespan. It has been reported that in adult flies, RNAi knockdown of Atg7 by Geneswitch-Actin-Gal4 did not show reduced lifespan [44]. This discrepancy may be due to the different Gal4 drivers used and that the knockdown of Atg7...
occurring only during adulthood is insufficient to cause shortened lifespan since autophagy activity is known to be tightly regulated during development.

Yet we cannot exclude that chaperone-mediated autophagy (CMA) is involved in the connection between Hsp27 and Atg7. CMA is a specific cargo delivery process to the lumen of the lysosome, mediated by Hsc70, Hsp90, and the lysosome-associated membrane protein type 2A (LAMP-2A) [45, 46]. However, a recent study in Drosophila shows that the co-chaperone Starvin assists in the coordination of Hsc70 and HspB8 through chaperone-assisted selective autophagy, which is distinct from CMA, to depose damaged filamin for muscle maintenance [47]. It is possible that Hsp27 may function through chaperone-assisted selective autophagy linking to Atg7.

Conclusion
In summary, our findings shed new insight in the linkage of Hsp27 to Atg7 in the regulation of eye morphology, polyglutamine toxicity, and lifespan. The information provides a new aspect in the understanding how Hsp27 may connect to Atg7 to modulate certain physiological functions.

Additional files

Additional file 1: Table S1. A summary of lifespan by the knockdown of Hsp27 in Drosophila.

Additional file 2: Table S2. A summary of lifespan by the overexpression or knockdown of Atg7 in Drosophila.

Additional file 3: Table S3. A summary of starvation stress response by overexpression of Atg7 in Drosophila.

Additional file 4: Table S4. A summary of lifespan resulting from simultaneous overexpression and knockdown of different combinations of Atg7 and Hsp27 in Drosophila.

Additional file 5: Figure S1. The flies with simultaneous overexpression of Hsp27 and knockdown of Atg7 display better climbing activity than those with overexpression of Hsp27 and knockdown of Atg7 under paraquat-induced oxidative stress. The climbing index for each strain: appl-Gal4/+ (the control fly), 21.8 ± 0.02% (n = 195); UAS-hsp27/+; appl-Gal4/UAS-hsp27[290], 44.7 ± 0.02% (n = 123); UAS-atg7/+; appl-Gal4/UAS-hsp27[290], 42.4 ± 0.01% (n = 175). (p is the total fly number from the four independent assays). (**p < 0.01, ***p < 0.001).

Abbreviations
Atg: autophagy-related gene; Hsp: heat shock protein.

Competing Interests
The authors declare that they have no competing interests.

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Author details
1Institute of Biotechnology, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, HsinChu, 30013, Taiwan. 2Department of Life Science, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, HsinChu, 30013, Taiwan. 3Institute of Biological Chemistry, 128, Section 2, Academia Road, Nankang, Taipei, 115, Taiwan. 4Institute of Neuroscience, National Chengchi University, 64, Section 2, Zhi-Nan Road, Taipei, 11605, Taiwan. 5Second Section of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital at Linkou, Chang Gung University College of Medicine, Taoyuan, Taiwan.

Authors’ contributions
S-F. Chen, M-L. Kang, Y-C. Chen, H-W. Tang, C-W. Huang, W-H. Li, and C-P. Lin carried out the experiments and analyzed the data; P-Y. Wang, G-C. Chen and H-D. Wang designed the experiments, analyzed the data, and together with C-Y. Wang discussed the data and wrote the manuscript. All authors read and approved the final manuscript.

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