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# The *miR-58* microRNA family is regulated by insulin signaling and contributes to lifespan regulation in *Caenorhabditis elegans*

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microRNAs regulate diverse biological processes such as development and aging by promoting degradation or inhibiting translation of their target mRNAs. In this study, we have found that the *miR-58* family microRNAs regulate lifespan in *C. elegans*. Intriguingly, members of the *miR-58* family affect lifespan differently, sometimes in opposite directions, and have complex genetic interactions. The abundances of the *miR-58* family miRNAs are up-regulated in the long-lived *daf-2* mutant in a *daf-16*-dependent manner, indicating that these miRNAs are effectors of insulin signaling in *C. elegans*. We also found that *miR-58* is regulated by insulin signaling and partially required for the lifespan extension mediated by reduced insulin signaling, germline ablation, dietary restriction, and mild mitochondrial dysfunction. We further identified the *daf-21*, *ins-1*, and *isw-1* mRNAs as endogenous targets of *miR-58*. Our study shows that miRNAs function in multiple lifespan extension mechanisms, and that the seed sequence is not the dominant factor defining the role of a miRNA in lifespan regulation.

***miR-58* family, insulin signaling, lifespan, *C. elegans***

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## INTRODUCTION

microRNAs (miRNAs) are posttranscriptional regulators of gene expression in diverse biological processes (Alvarez-Saavedra and Horvitz, 2010; Ambros, 2004; Kloosterman and Plasterk, 2006; Lee et al., 1993). In *Caenorhabditis elegans*, 250 miRNA precursors have been annotated and they are predicted to generate a total of 434 mature miRNAs (http://www.mirbase.org/). Although functionally uncharacterized for most of them, many miRNAs are differentially expressed during development (Kato et al., 2009) or during aging (Ibáñez-Ventoso et al., 2006). Adult-specific knock-down of the argonaute-like gene *alg-1*, which is necessary for miRNA maturation and function, results in a shortened lifespan (Kato et al., 2011). Several miRNAs, including *lin-4*, *mir-71*, and those belonging to the *let-7* family, have been shown to be longevity regulators in *C. elegans* (Boehm and Slack, 2005; Boulias and Horvitz, 2012; Shen et al., 2012; Wang et al., 2017).

The *miR-58* family is composed of five members, *miR-58*, *miR-80*, *miR-81*, *miR-82*, and a recently discovered *miR-1834* (Figure S1 in Supporting Information) (Friedländer et al., 2008; Lau et al., 2001). They are the homologs of *bantam*, a *Drosophila* miRNA that controls cell proliferation and apoptosis, and *hsa-miR-143*, a human miRNA involved in cancer progression (Brennecke et al., 2003; Chen et al., 2009; Clapé et al., 2009; Pagliuca et al., 2013). *miR-58* is reported to be the most abundant miRNA in *C. elegans*, constituting nearly half of the total amount of miRNAs in a

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worm, and it is expressed throughout the life cycle of *C. elegans* (Kato et al., 2009). The summed quantity of *miR-80*, *miR-81*, and *miR-82* is less than 1.5% of the total amount of miRNAs (Kato et al., 2009). *miR-1834* was hardly detectable (Kato et al., 2009; Warf et al., 2011). Worms lacking a single member of the *mir-58* microRNA family do not display obvious defects (Miska et al., 2007), but those lacking four members of the *mir-58* family, including *mir-58*, *mir-80*, *mir-81*, and *mir-82*, have a smaller body size and are defective in locomotion, pharyngeal pumping, dauer formation and egg-laying (Alvarez-Saavedra and Horvitz, 2010). This indicates that the *miR-58* family of miRNAs have important and wide-ranging functions, and there is functional redundancy between family members (Subasic et al., 2015).

Previous studies have revealed that either a single-gene deletion of *mir-58* or a triple deletion of *mir-80*, *mir-81* and *mir-82* (abbreviated as *mir-80/81/82*, same below) shortens the lifespan of wild-type (WT) worms by 20% (Boulias and Horvitz, 2012). In contrast, deletion of *mir-80* extends lifespan by 5%–18% (Vora et al., 2013). Interestingly, the expression levels of both *miR-58* and *miR-80* decrease with age (Ibáñez-Ventoso et al., 2006; Kato et al., 2011; Lucanic et al., 2013). These results suggest that the *miR-58* family may participate in lifespan regulation.

Regulation of aging by the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway, referred to as the insulin/insulin-like growth factor 1 signaling pathway (IIS pathway), is highly conserved across phylogeny (Piñero González et al., 2009). In *C. elegans*, the insulin/IGF-1 receptor DAF-2 negatively regulates the activity of DAF-16, a Forkhead transcription factor in the FoxO subgroup, through several kinases including AGE-1, AKT-1, and AKT-2 (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997; Paradis and Ruvkun, 1998; Pierce et al., 2001). Partial loss-of-function mutations of *daf-2* double the WT lifespan, whereas the *daf-16* null mutants live slightly shorter than WT (Dillin et al., 2002a; Gems et al., 1998; Kenyon et al., 1993). The extraordinary longevity of the *daf-2* mutants completely depends on *daf-16* because the *daf-2; daf-16* double mutants have a lifespan just like those of the *daf-16* single mutants (Kenyon et al., 1993; Lee et al., 2001; Ogg et al., 1997). Previous studies have shown that key components of the IIS pathway are regulated by miRNAs. For example, the *let-7* family of miRNAs *miR-48*, *miR-84*, *miR-241*, and *let-7* itself target the mRNAs of *akt-1* or both *akt-1* and *akt-2* (Shen et al., 2012; Wang et al., 2017).

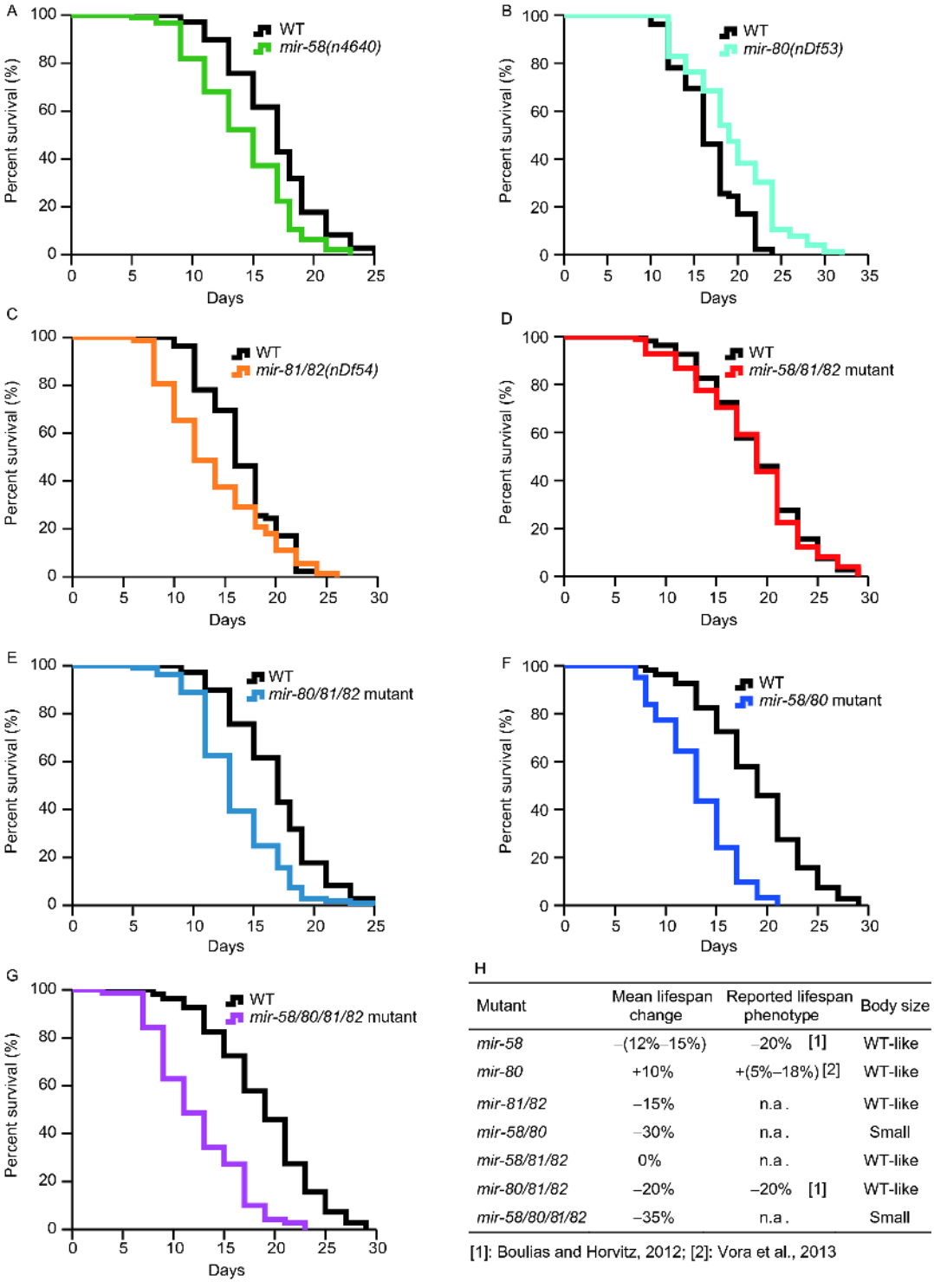
In this study, we found that the abundances of *miR-58*, *miR-80*, *miR-81*, and *miR-82* increased significantly in the long-lived *daf-2* mutant worms in a *daf-16*-dependent manner. Lifespan assays showed that deleting one or more members of the *mir-58* miRNA family altered *C. elegans* lifespan in an intriguingly complex way. Further studies suggested that *miR-58* plays a role in multiple well-known lifespan extension pathways, and the endogenous targets of *miR-58* include *daf-21*, *ins-1*, and *isw-1*.

## RESULTS

### Members of the *miR-58* microRNA family affect lifespan differently and have complex genetic interactions

To investigate the involvement of the *miR-58* microRNA family (Figure S1 in Supporting Information) in lifespan regulation, we performed lifespan assays on the mutant strains lacking one or more members of the *mir-58* family (Table S1 in Supporting Information). Deleting *mir-58* alone shortened lifespan by 12%–15%, whereas deleting *mir-80* alone extended lifespan by about 10% (Figure 1A and B; Table S2 in Supporting Information), both of which agree with the previously reported lifespan phenotypes (Boulias and Horvitz, 2012; Vora et al., 2013). The *nDf54* allele deletes both *mir-81* and *mir-82*, two adjacent genes whose products differ by only one nucleotide in the non-seed region (Figure S1 in Supporting Information). *mir-81* and *mir-82* are probably the result of a recent gene duplication event and likely to have redundant functions. Practically, it is difficult to measure their quantities separately, so we analyzed them as one, referred to as *mir-81/82* in this study. Loss of *mir-81/82* shortened lifespan by about 15% (Figure 1C; Table S2 in Supporting Information).

Having found out that individual members of the *miR-58* family affects lifespan differently, we further investigated the interactions between the family members by analyzing the double or triple knock-out mutants. To our surprise, deleting both *mir-58* and *mir-81/82* resulted in a WT-like lifespan (Figure 1D; Table S2 in Supporting Information), even though deleting either shortened lifespan (Figure 1A and C; Table S2 in Supporting Information). This indicates a mutual suppression effect. Deletion of *mir-80/81/82* shortened lifespan by 20% (Figure 1E; Table S2 in Supporting Information), which is consistent with a previous study and shows that the pro-longevity effect of *mir-80(null)* fails to suppress the lifespan shortening effect of *mir-81/82(null)*. The *mir-80/81/82* mutant worms are not obviously sick, suggesting that enhancement of the short lifespan phenotype of the *mir-81/82* mutant by *mir-80(null)*, which extends WT lifespan, is more likely the result of a complex genetic interaction rather than that of general sickness. Lastly, deleting both *mir-58* and *mir-80* or deleting *mir-58*, *mir-80*, *mir-81*, and *mir-82* made the worms small (Figure 1H; Figure S2 in Supporting Information) and sick, and shortened lifespan by 30%–35% (Figure 1F and G; Table S2 in Supporting Information). In fact, the *mir-58/80* double mutant displayed all the defects reported for the *mir-58/80/81/82* mutant, including a small body size, uncoordinated movement, laying late-stage embryos, and inability to form dauers (Alvarez-Saavedra and




H

| Mutant | Mean lifespan change | Reported lifespan phenotype | Body size |
|---|---|---|---|
| *mir-58* | –(12%–15%) | –20% [1] | WT-like |
| *mir-80* | +10% | +(5%–18%) [2] | WT-like |
| *mir-81/82* | –15% | n.a. | WT-like |
| *mir-58/80* | –30% | n.a. | Small |
| *mir-58/81/82* | 0% | n.a. | WT-like |
| *mir-80/81/82* | –20% | –20% [1] | WT-like |
| *mir-58/80/81/82* | –35% | n.a. | Small |

[1]: Boulias and Horvitz, 2012; [2]: Vora et al., 2013

**Figure 1** Lifespans of *C. elegans* mutants lacking one or more members of the *mir-58* family. A, Loss of *mir-58* shortened WT lifespan by about 15% ($P$<0.001). B, Loss of *mir-80* extended WT lifespan by about 10% ($P$<0.01). C, Loss of *mir-81/82* shortened WT lifespan by about 15% ($P$<0.001). D, The *mir-58/81/82* triple mutant had a WT-like lifespan ($P$>0.05). E, The *mir-80/81/82* mutant lived about 20% shorter than WT animals ($P$<0.001). F and G, Loss of *mir-58/80* (F) or *mir-58/80/81/82* (G) significantly shortened WT lifespan by about 30% ($P$<0.001). H, Summary of the lifespan and body size phenotypes of the *mir-58* family mutants. n.a., not available. Lifespan was assayed at 20°C. $P$-values were calculated by log-rank tests.

Horvitz, 2010). The above results showed that despite sequence similarity, members of *miR-58* family affect lifespan differently and have complex genetic interactions.

### There are no obvious compensatory effects among the *miR-58* family members

To find out whether some of the perplexing phenotypes ob-

served might be the result of over-compensation, that is, the loss of one or more members induces overexpression of the remaining family member(s), we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis on the different mutants. As shown in Figure 2, we did not find strong evidence of over-compensation, as deleting one or more *mir-58* family miRNAs did not change the expression of the remaining ones by more than 50%, nor at a significant level of $P<0.01$ (Figure 2; Table S3 in Supporting Information).

### *miR-58* is regulated by IIS pathway and partially required for the lifespan extension by reduced IIS signaling, germline ablation, dietary restriction, and mild mitochondrial dysfunction

In *C. elegans*, a reduction of insulin/IGF-1 signaling induces the most robust lifespan extension phenotype. To investigate whether the *mir-58* microRNA family is connected in some way to the IIS pathway, we quantified the expression levels of the family members in the WT N2 strain, the long-lived *daf-2*(*e1370*) mutant, the short-lived *daf-16(mu86)* mutant, and the similarly short-lived *daf-2(e1370); daf-16(mu86)* double mutant by qRT-PCR. We found that the *miR-58* family members were significantly up-regulated in the *daf-2* mutant in a *daf-16*-dependent manner (Figure 3A; Table S3 in Supporting Information). *miR-1834* was not detected in any of these strains (data not shown).

Next, we focused on one family member *miR-58* and investigated whether it contributes to lifespan regulation by IIS. *miR-58* is reported to be the most abundant miRNA in *C. elegans* and outweighs its family members by more than 20-fold in quantity (Kato et al., 2009). Consistent with our hypothesis that the up-regulation of *miR-58* contributes to the *daf-2* longevity, the *daf-2; mir-58* double mutant lived about 15% shorter than the *daf-2* mutant (Figure 3B, Tables S1 and S2 in Supporting Information). The shortening of the *daf-2* lifespan by *mir-58(null)* is probably not due to a general sickness effect of *mir-58(null)*, because it did not shorten the *daf-16(null)* lifespan (Figure 3C, Tables S1 and S2 in Supporting Information). In fact, the *daf-16; mir-58* double mutant lived longer than the *daf-16* mutant ($P<0.001$).

Besides IIS, additional mechanisms exist to regulate aging in *C. elegans* such as germline signaling (Hsin and Kenyon, 1999), nutrient sensing (Kaeberlein et al., 2006; Lee et al., 2006), and signals from the mitochondria (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003). We asked whether *miR-58* is involved in lifespan extension through these mechanisms. To find out, we crossed the *mir-58(null)* allele into three long-lived mutants including *glp-1(e2144)*, which lacks the germline at 25°C, *eat-2(ad1113)*, which genetically mimics dietary restriction, and *isp-1(qm150)*, which causes mild mitochondrial dysfunction (Table S1 in Supporting Information). As shown in Figure 3D–F, *mir-58(null)* comprised the longevity of all three mutants. These results showed that *mir-58* is at least partially required for lifespan extension through these mechanisms.

### *daf-21*, *ins-1* and *isw-1* are endogenous mRNA targets of *miR-58*

To understand how *miR-58* functions in lifespan regulation, particularly in IIS-mediated lifespan extension, we sought to identify the target genes of *miR-58*. Among the several hundreds of *miR-58* targets predicted by TargetScan (Jan et al., 2011; Lewis et al., 2005), we selected 13 candidates for further study (Figure 4A). Two of them, *ins-1* and *ins-17*, encode insulin-like peptides in *C. elegans* (Pierce et al., 2001). RNAi of *daf-21*, which encodes a HSP90 chaperone, partially suppresses the longevity caused by lowering IIS (Morley and Morimoto, 2004). For *dbl-1*, *egl-2*, *mkk-4*, *ogt-1*, and *scd-2*, it has been shown that their loss-of-function mutations shorten WT lifespan (Love et al., 2010; Mallo et al., 2002; Shen et al., 2007; Ventura et al., 2009). For the remaining five, *flh-2*, *gfl-1*, *hmg1.1*, *lin-52*, and *isw-1*, either their protein abundance (Jovanovic et al., 2012; Jovanovic et al., 2010) or mRNA abundance (Jovanovic et al., 2012), or both, increased upon deletion of *mir-58*, fitting with the expectation that a miRNA negatively regulates the stability or the translation rate of its target mRNA in most cases (Bartel, 2004). To test whether the above 13 candidates are direct targets of *miR-58*, we designed a dual-luciferase assay. As shown in Figure 4B, for each candidate target gene, its 3′-untranslated region (3′-UTR) was placed downstream of the firefly luciferase coding sequence, and a rellina luciferase coding sequence in the same plasmid served as an internal control. The engineered plasmid was transfected into HEK293T cells with or without a miRNA mimic. The miRNA mimic is designed to mimic endogenous mature miRNA molecules when transfected into cells. As a positive control, the *let-7* mimic effectively repressed the firefly luciferase carrying the 3′-UTR of *daf-12*, a well-established *let-7* target gene (Figure S3 and Table S4 in Supporting Information) (Jovanovic et al., 2010). We then examined the interaction between each of the 13 candidates and *miR-58*, and found that a fusion of the 3′-UTR of *daf-21*, *gfl-1*, *ins-1*, or *isw-1* to the firefly luciferase mRNA significantly decreased the ratio of firefly/rellina activity upon co-transfection with *miR-58* mimic, suggesting that *miR-58* may directly bind to these 3′-UTR sequences (Figure 4C; Table S4 in Supporting Information). Further repeat experiments confirmed the results (Figure 4D). To be sure, we quantified the expressions of *daf-21*, *gfl-1*, *ins-1*, and *isw-1* by qRT-PCR in

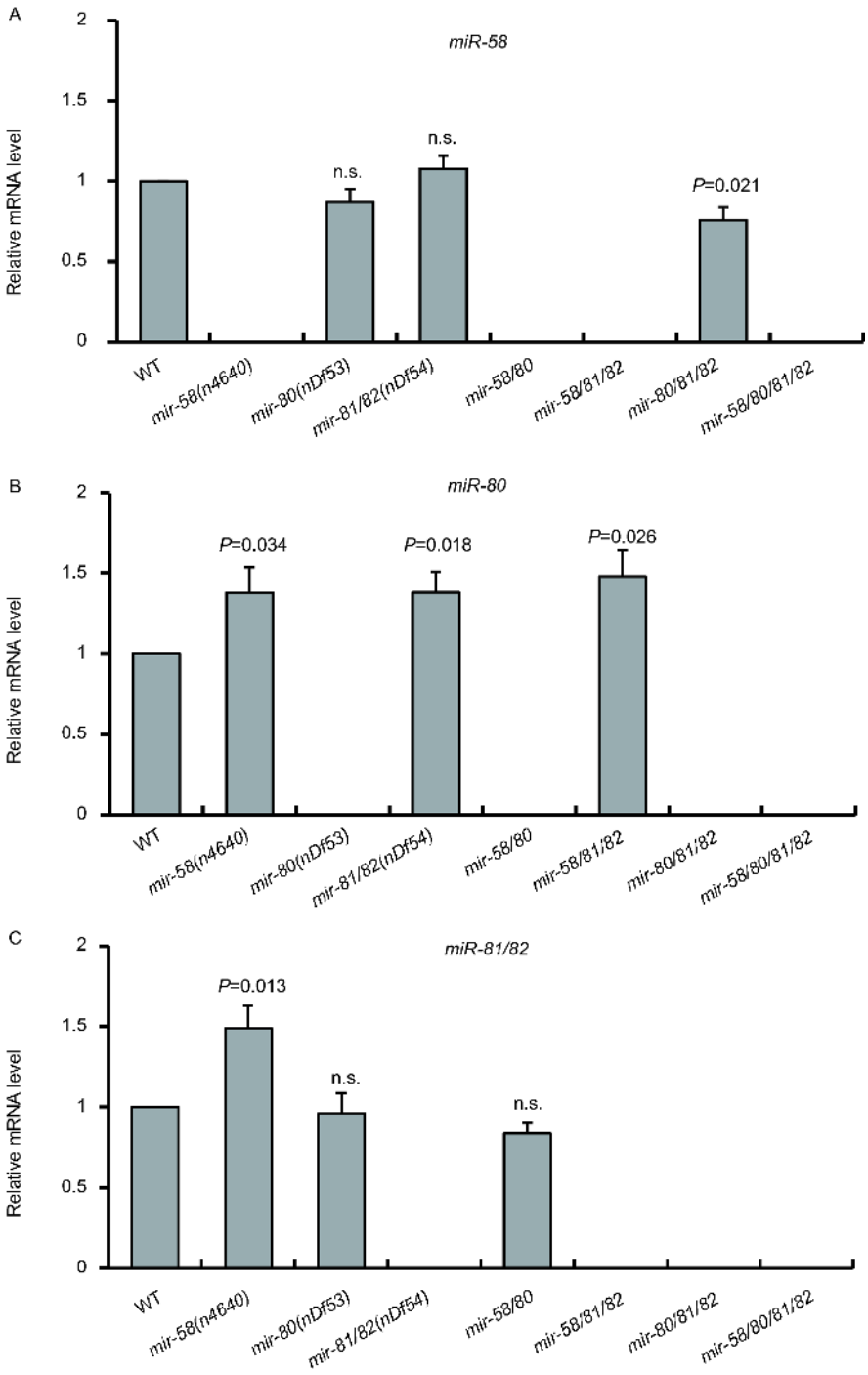



**Figure 2** (Color online) Relative abundance changes of the indicated *miR-58* family miRNA when other family members are deleted. A–C, Abundance of *miR-58*, *miR-80*, and *miR-81/82*, respectively, normalized to the WT level. The results are represented as mean±SD from three independent qRT-PCR experiments. Student's *t*-tests were used to calculate the *P*-values. n.s., not significant, $P>0.05$.

WT, the *mir-58*, and the *mir-58/80/81/82* mutant worms (Figure 4E; Table S3 in Supporting Information). The mRNA levels of *daf-21*, *ins-1*, and *isw-1* indeed increased markedly in the absence of *mir-58*, supporting the idea that these three genes are endogenous targets of *miR-58* in *C. elegans*. Moreover, the expression levels of *daf-21* and *ins-1* were further increased in the *mir-58/80/81/82* mutant compared with that of the *mir-58* mutant, suggesting that the *miR-58* family members act additively to regulate the expressions of these two targets. *gfl-1*, which encodes a putative transcription factor, was not confirmed as an endogenous target gene of *miR-58* in this experiment.

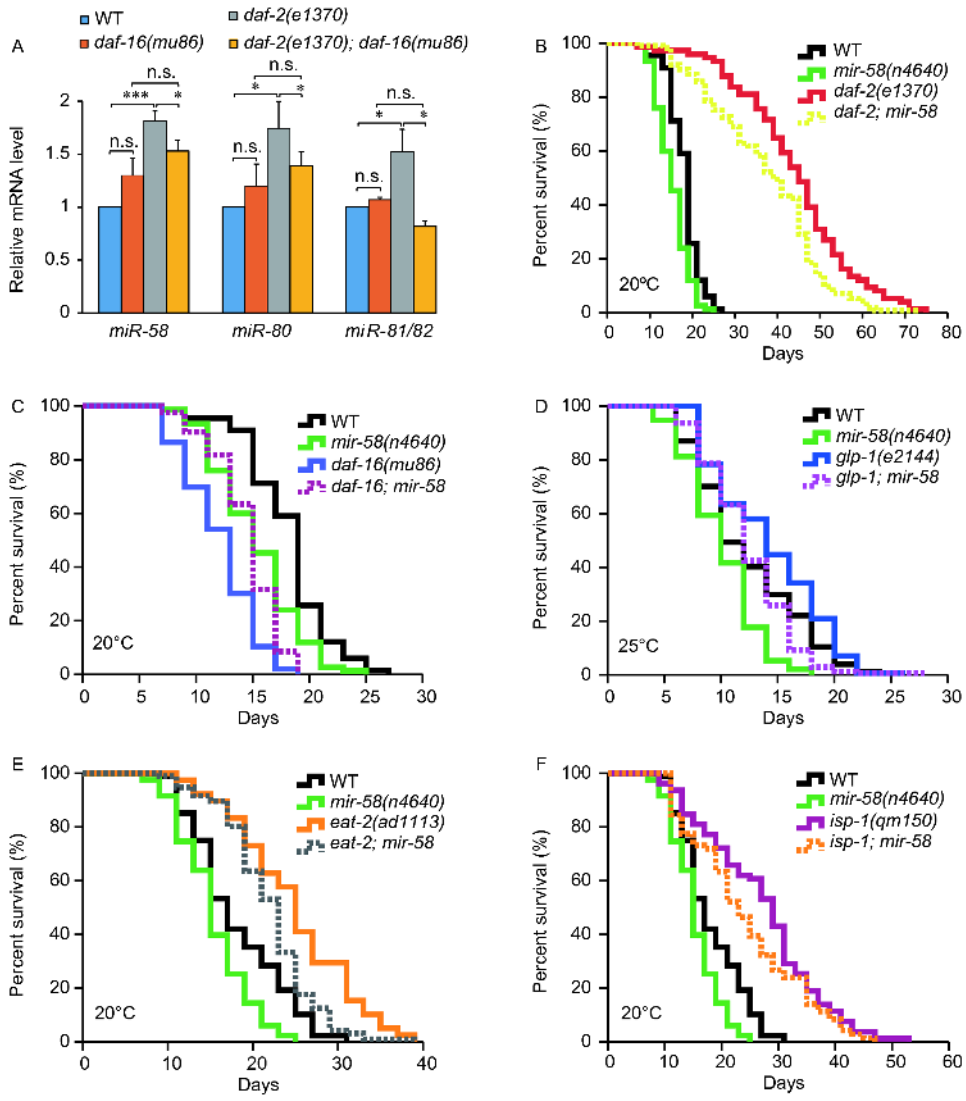



**Figure 3** *miR-58* is regulated by *daf-2* and contributes to the longevity of the *daf-2* mutant and three other mutants. A, The expression levels of *miR-58*, *miR-80*, and *miR-81/82* increased significantly in the *daf-2* mutant in a *daf-16*-dependent manner. The results represent three biological replicates, shown as mean±SD. Student's *t*-tests were used to calculate the *P*-values. ***, $P<0.001$; *, $P<0.05$; n.s., not significant, $P>0.05$. B, *mir-58(n4640)* shortened the *daf-2 (e1370)* lifespan by about 15% ($P<0.001$). C, The *daf-16; mir-58* double mutant lived longer than the *daf-16(mu86)* mutant ($P<0.001$). D–F, *mir-58(n4640)* suppressed the long lifespan of *glp-1(e2144)* (D), *eat-2(ad1113)* (E) and *isp-1(qm150)* (F) mutants by about 12% ($P<0.001$), 12% ($P<0.001$) and 11% ($P<0.05$), respectively. *P*-values were calculated by log-rank tests.

### The abundances of *daf-21* and *gfl-1* are decreased in the long-lived *daf-2* mutant in a *daf-16*-dependent manner

Since the long-lived *daf-2* mutant worms express more *miR-58* than WT and *daf-16* mutant worms (Figure 3A), we predicted that the amount of the *miR-58* target mRNAs *daf-21*, *ins-1*, and *isw-1* should decrease. This was indeed the case for *daf-21* but not for *ins-1* and *isw-1* (Figure 5A; Table S3 in Supporting Information). The *gfl-1* mRNA, which was not confirmed as an endogenous target of *miR-58* by comparing WT and the *mir-58* mutant (Figure 4E), showed significant abundance decrease in the *daf-2* mutant just like *daf-21* did, and the decrease was also dependent on *daf-16*. Based on the data above, we conclude that *daf-21*, *ins-1*, and *isw-1*, which encode a chromatin remodeler gene, are bona fide targets of *miR-58*.

Despite the efforts in this study, our findings fall short of explaining how *miR-58* regulates lifespan. Extrapolating from the reported short lifespan of the *isw-1* mutant (Matilainen et al., 2017), the long lifespan of *ins-1* overexpression (Pierce et al., 2001), and the suppression of the *age-1* mutant longevity by *daf-21* RNAi (Morley and Morimoto, 2004), we would expect the *mir-58* mutant to be long-lived, but this is opposite to the observed short lifespan phenotype. One interpretation for this discrepancy is that the reported lifespan phenotypes were caused by globally knocking-down or overexpressing these genes in *C. elegans*, but regulation of these genes by *miR-58* may be limited to certain tissues or

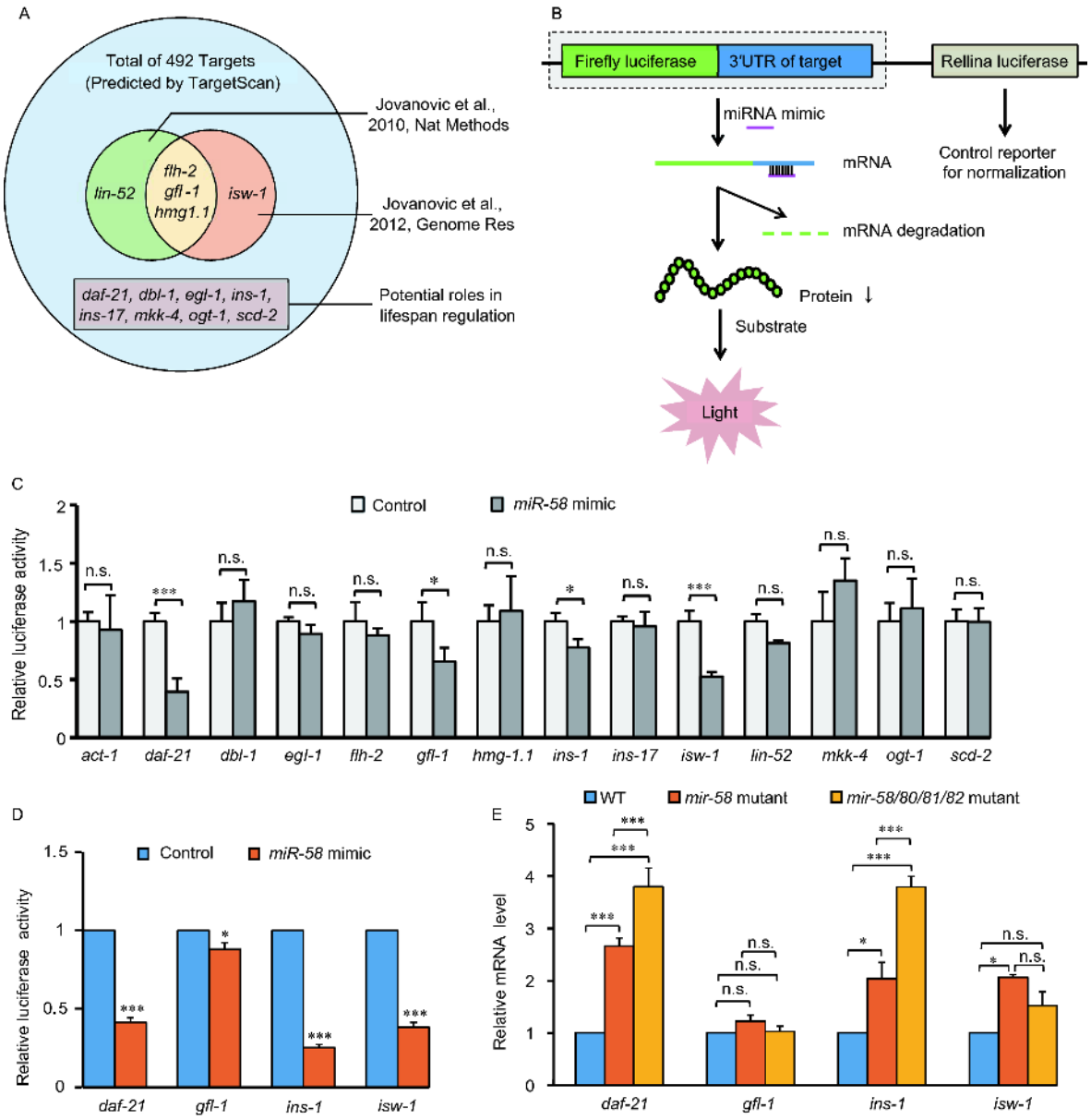



**Figure 4** Identification of *miR-58* targets. A, 13 candidate targets of *miR-58* were selected by combining computational predictions with published experimental data. B, A diagram showing the working mechanism of the dual-luciferase assay. C, Luciferase assay to examine the interactions between *miR-58* and the 3′-UTRs of 13 candidate targets in cultured cells. 3′-UTR of *act-1* was used as negative control. D, Verification of four candidate targets identified from (C) by three independent dual-luciferase assays in cultured cells. E, The *daf-21*, *ins-1*, and *isw-1* mRNA levels were up-regulated in the *mir-58* mutant as quantified by qRT-PCR (three biological replicates). Mean±SD are shown in the bar graphs. Significant statistical differences were calculated by Student's *t*-tests. ***, $P<0.001$; *, $P<0.05$; n.s., not significant, $P>0.05$.

developmental stages. More likely, there may be additional targets mediating the effect of *miR-58* on lifespan (Figure 5B). Similarly, we think that there are yet-to-be-identified *miR-58* targets in the *daf-2* mutant.

## DISCUSSION

Previous studies have tried to characterize the physiological functions and corresponding targets of the *miR-58* family microRNAs. de Lucas et al. identified four genes *dbl-1*, *daf-1*, *daf-4*, and *sma-6* of the TGF-β pathways as the targets regulated by the *miR-58* family (de Lucas et al., 2015), supporting the idea that the *miR-58* family members are regulators of growth and stress response, which agrees with the role of *bantam* in *Drosophila* (Brennecke et al., 2003). Lozano et al. found that the *miR-58* family suppresses the expression of *sta-1* to regulate dauer formation (Lozano et al., 2016). Pagano et al. reported that the *miR-58* family plays important roles in switching off the expression of *pmk-2* in non-neuronal tissues (Pagano et al., 2015). More recently, Ryan et al. demonstrated that the *miR-58* family cooperates with the *miR-35* family to control *egl-1* expression in the

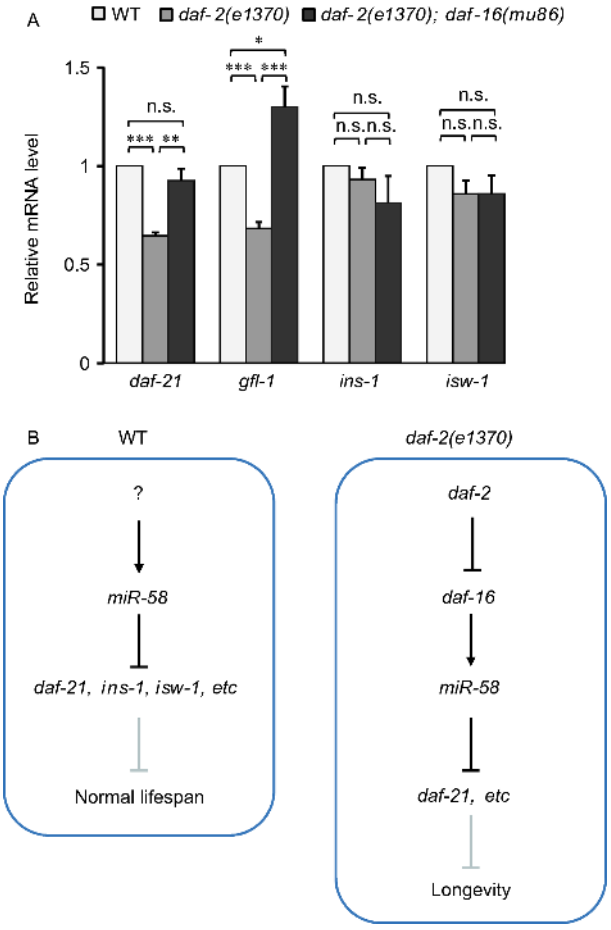



**Figure 5** (Color online) Decrease of the *daf-21* and *gfl-1* mRNA levels in the *daf-2* mutant in a *daf-16*-dependent manner. A, qRT-PCR of the *daf-21*, *gfl-1*, *ins-1*, and *isw-1* mRNAs in the WT, the *daf-2* mutant, and the *daf-2; daf-16* double mutant. The results are normalized to the WT values and shown as mean±SD. Significant statistical differences were calculated by Student's *t*-tests. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; n.s., not significant, $P>0.05$. B, Models of how *miR-58* contributes to lifespan regulation.

precursors of the cells that are programmed to die during embryogenesis (Sherrard et al., 2017). In this study, we show that *daf-21*, *ins-1*, and *isw-1* are *miR-58* targets, and members of the *miR-58* miRNA family all play a role in lifespan regulation. In particular, *mir-58* is at least partially required for lifespan extension by IIS, germline ablation, dietary restriction, and mild mitochondrial dysfunction. Our results add to the previous findings and demonstrate that the *miR-58* family miRNAs regulate diverse cellular activities and have many mRNA targets.

One notable finding in this study is that the different members of the *miR-58* miRNA family affect lifespan differently, even in opposite directions, and have complex, unexpected genetic interactions between one another (Figure 1). For example, two short-lived mutants produced WT-like lifespan when crossed together (the *mir-58/81/82* mutant), and a cross between a long-lived mutant and a short-lived mutant produced a double mutant (*mir-58/80*) that lived much shorter than either parent strain. The 5′-end nucleotides 2–8 of miRNAs, the so-called "seed" region, is crucial for target recognition (Bartel, 2004; Brennecke et al., 2005; Lai, 2002), and the *C. elegans miR-58* family members share exactly the same seed region sequence except *miR-58*, which has a "G" instead of "A" at position 8 (Figure S1 in Supporting Information). Clearly, the seed region cannot explain the phenotype difference among the *miR-58* family members. It has been shown for *lin-4* and *let-7* miRNAs, nucleotides outside the seed region play important roles in suppressing the expression of *lin-41* and *lin-14*, targets of *lin-4* and *let-7*, respectively (Ha et al., 1996; Reinhart et al., 2000; Zhang et al., 2015). We propose that for the *miR-58* family miRNAs, the non-seed region is critically involved in target recognition and responsible for some of the differences seen in the mutants. Also possible is that the *miR-58* family members may be expressed in different cells, which gives them access to different pools of mRNAs to regulate and thus different functions.

The targets that mediate the effect of *miR-58* on lifespan remain to be elucidated. The candidate pool from which we screened for *miR-58* targets was restricted to the ones predicted by TargetScan, with additional filtering criteria applied such as expected abundance changes of the target mRNAs or the corresponding proteins, whether linked to IIS or not, or with a reported lifespan phenotype. We probably missed relevant targets of *miR-58* in lifespan regulation from the outset. In the future, additional targets predicted by TargetScan could be screened. Another reason for missing a true target of *miR-58* may have to do with the computational algorithms that predict miRNA targets, because they are most concerned with the match between the seed region of a miRNA and a conserved 7- or 8-nt sequence in the 3′-UTR of a candidate target mRNA. However, studies have revealed that miRNAs also target noncanonical sites in the coding sequence or the 5′-UTR (Forman et al., 2008; Helwak et al., 2013; Zhou et al., 2009). Lastly, *miR-58* may have many targets; if the lifespan phenotype of *mir-58* mutant reflects the balance between many affected targets, it would be difficult to dissect the contribution of individual targets.

## MATERIALS AND METHODS

### Strains and culture conditions

The following strains were obtained from Caenorhabditis Genetics Centre (CGC): wild-type N2 strain (Bristol), MT15024 *mir-58(n4640)* IV, MT14128 *mir-80(nDf53)* III; *mir-81/mir-82(nDf54)* X, MT15563 *mir-80(nDf53)* III; *mir-58(n4640)* IV; *mir-81/mir-82(nDf54)* X, CB1370 *daf-2 (e1370)* III, CF1038 *daf-16 (mu86)* I, DA1113 *eat-2(ad1113)* II, CB4037 *glp-1(2144)* III and MQ887 *isp-1 (qm150)* IV. MT15024 was outcrossed with N2 for seven times before any test was performed.

The following mutant strains were generated in this study: MQD1162 *mir-80 (nDf53)* III, MQD1163 *mir-81/82(nDf54)* X, MQD1262 *mir-80(nDf53)* III; *mir-58(n4640)* IV, MQD1308 *mir-58(n4640)* IV; *mir-81/82(nDf54)* X, MQD1195 *daf-2(e1370)* III; *mir-58(n4640)* IV, MQD1196 *daf-16(mu86)* I; *mir-58(n4640)* IV, MQD1200 *glp-1(2144)* III; *mir-58(n4640)* IV, MQD1203 *eat-2(ad1113)* II; *mir-58 (n4640)* IV and MQD1208 *mir-58(n4640)* IV; *isp-1(qm150)* IV. Among these strains, MQD1162 *mir-80 (nDf53)* III and MQD1163 *mir-81/82(nDf54)* X were isolated by crossing MT14128 with N2, and the double or triple mutants were made using standard genetic methods.

The genotypes of all strains were confirmed by PCR or PCR followed by sequencing. Primer sequences used for genotyping are listed in Table S1 in Supporting Information.

Strains were maintained on agar plates containing standard nematode growth media (NGM) seeded with *Escherichia coli* OP50 at 20°C according to standard procedures (Brenner, 1974), except for CB1370 and CB4037 that were maintained at 15°C.

### Lifespan analysis

All lifespan assays were performed at 20°C, unless otherwise indicated. Strains were synchronized by allowing 40 gravid adults to lay eggs on OP50-seeded NGM plates for 3 h at 20°C and then picking off adult worms. Worms were allowed to grow for several days until they reached L4 larval stage. Approximately 150 L4 worms were then transferred to ten fresh OP50-seeded NGM plates (15 worms/plate). Day 1 of the lifespan was the point when the worms reached young adult stage. The animals were transferred to new plates every 2 days until the end of reproductive period. Live worms were scored every 2 days, and a worm was scored as death if it showed no response to the touch with a worm pick. Animals that exploded, bagged, crawled off the plate or became contaminated were censored from the analysis. IBM SPSS Statistics 20 software was used to perform statistical analysis, and *P*-values were calculated using the log-rank (Mantel-Cox) method (Table S2 in Supporting Information).

### qRT-PCR analysis

To obtain synchronized worms for qRT-PCR experiments, eggs were obtained by bleaching gravid adults. After hatching, the synchronized L1 larvae were cultured on high-growth plates seeded with OP50 at 20°C until they reached young adult stage. Worms were harvested and used for total RNA isolation using TRIZOL (Invitrogen, USA).

We adopted stem-loop qRT-PCR method to quantify the expressions of miRNAs (Chen et al., 2005). Each stem-loop reverse transcription (RT) primer is composed of a 44-nucleotide stem-loop sequence, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC-3′ (Shen et al., 2012) followed by a 6-nucleotide sequence that are exactly complementary to the 3′-end of the mature miRNA (Table S3 in Supporting Information). cDNA was prepared by using the MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) and a mixture of stem-loop RT primers. Then a miRNA-specific forward primer and a universal reverse primer (mir-R) were used for qPCR. qRT-PCR was performed with SYBR Green master mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). To quantify mRNA targets, cDNA was generated from total RNA by Primescript RT Reagent Kit with random hexamers (TaKaRa, Japan), and the qPCR reaction was the same as that of miRNA quantification. Three technical replicates were performed for each reaction. U18 snoRNA and *pmp-3* were used as the internal control for miRNA and mRNA quantification, respectively. All primer sequences used in qRT-PCR experiments are listed in Table S3 in Supporting Information.

### Plasmids construction

The 3′-UTR sequences of *miR-58* target genes and control genes were retrieved from the Wormbase (http://www.wormbase.org/). These 3′-UTR sequences were then amplified from N2 genomic DNA by PCR and cloned directly downstream the firefly luciferase gene in pmirGLO vector (Promega, USA) by *Sac* I/*Sbf* I sites using the Infusion Cloning Kit (Clontech, USA). The primers used for PCR amplification of the 3′-UTRs are listed in Table S4 in Supporting Information.

### Cell culture and dual-luciferase assays

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 μg $mL^{-1}$ penicillin/streptomycin in 5% $CO_2$ at 37°C. For dual-luciferase assay, we grew HEK293T cells in DMEM with 10% FBS but without antibiotics and seeded them at a density of $1\times10^4$ cells per well into 96-well plates. 24 h later, cells were transfected in triplicate with Lipofectamine 2000 (Invitrogen), 100 ng of the dual-luciferase vector in which the Firefly luciferase is tagged with the 3′-UTR of target candidates, and 100 nmol of *miR-58* mimic miRNA (purchased from RiboBio Co., Ltd, Guangzhou). The transfection experiment with *let-7* mimic miRNA and the dual-luciferase vector with *daf-12* 3′-UTR was carried out as a positive control for luciferase assay. Dual-luciferase reporter assay was performed 48 h post transfection using the Dual-Glo Luciferase Assay kit (Promega) according to the manufacturer's instructions. The luminescence was measured with a 2300 EnSpire Multilabel Plate Reader (PerkinElmer, USA). All the firefly luciferase readouts were first normalized with their corresponding rellina luciferase readouts, then

the resulting values were used to calculate the relative luciferase activity by considering luminescence values from the same 3′-UTR luciferase vector but transfected without *miR-58* mimic as "1".

**Compliance and ethics** *The author(s) declare that they have no conflict of interest.*

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## SUPPORTING INFORMATION

**Figure S1** Sequences of the *C. elegans miR-58* family members, their homologs *bantam* and *hs-mir-143* in *Drosophila melanogaster* (*D. m.*) and human, respectively.

**Figure S2** The body size of the *mir-58* family mutants. Only the *mir-58/80* and *mir-58/80/81/82* mutants are smaller than the WT animals.

**Figure S3** The *let-7* miRNA and its target *daf-12* served as a positive control for dual-luciferase assay.

**Table S1** Primers used for genotyping the worm strains

**Table S2** Summary of the lifespan data related to Figures 1 and 3

**Table S3** Primers used for miRNAs and mRNAs qRT-PCR

**Table S4** Primers used for amplifying the 3′-UTRs of miRNA targets in dual-luciferase assays

The supporting information is available online at http://life.scichina.com and https://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.