A Steroid Receptor–MicroRNA Switch Regulates Life Span in Response to Signals from the Gonad
Yidong Shen et al.
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T51, which silences Igf2r, and T16, which does not (Fig. 3A and fig. S7). The larger size of wild-type Airn is therefore unrelated to Igf2r silencing. The majority of FISH signals lay in the mid-nuclear plane, with both repressing and non-repressing Airn alleles showing a similar localization (Fig. 3B) and a similar relative position to the nucleoli (fig. S7). Together, these data indicate no organizational role for the Airn product in Igf2r silencing, thereby supporting claims (9) that Airn silences Igf2r and Slc22a3 by different mechanisms.

A prediction of a transcriptional overlap model is that the interfering promoter should impose repressor activity. To test this, we moved the Airn promoter ~700 base pairs before the Igf2r transcription start site (TSS) in ES cells that lack an endogenous paternal Airn promoter (16) (figs. S8 and S9). FAP (forward Airn promoter) cells contain the repositioned Airn promoter and the first 1.8 kb of the Airn IncRNA product (also present in the T3 and T16 alleles that do not silence Igf2r) in wild-type orientation, and express normal levels of Airn that overlap the paternal Igf2r promoter (Fig. 4A and fig. S9B). RAP (reverse Airn promoter) cells contain an inverted repositioned Airn promoter and do not transcribe Airn over the paternal Igf2r promoter. Undifferentiated ES cells showed biallelic Igf2r expression in FAP or RAP cells (fig. 4C and fig. S10B). Transcriptional interference models (18) predict suppression of the “sensitive” promoter by an “interfering” promoter, initially in the absence of repressive chromatin. The repressed FAP Igf2r promoter maintained features associated with active chromatin, such as a strong DNase I–hypersensitive site (Fig. 4D and fig. S12, A and B) and H3K4me3 (fig. S12C), similar to the active RAP Igf2r allele. The wild-type paternal Igf2r promoter is modified late in development by DNA methylation that is unnecessary for Igf2r repression in embryo or placenta (10) and by H3K9me3 (15, 19), which is unnecessary for Igf2r silencing in the placenta (9). The repressed Igf2r promoter remained free of DNA methylation (Fig. 4E), possibly due to the proximity of the repositioned Airn promoter CGI. Low-level H3K9me3 was less than on the silent Airn promoter by a factor of 10, similar to ratios in mouse embryonic fibroblasts (MEFs) (19), (Fig. 4F). Together, these data show that Airn transcriptional overlap interferes with functional RNAPII recruitment to the Igf2r promoter in the presence of active chromatin, supporting a model whereby Airn induces silencing by transcriptional interference (fig. S12D).

Collectively, our data demonstrate a role for Airn transcription, but not its spliced or unspliced lncRNA products, in silencing the Igf2r promoter. The demonstration that Igf2r silencing depends on Airn transcription reflects hallmark features of macro IncRNAs, such as inefficient splicing, extreme length, high repeat content, lack of conservation, and short half-life (15), which all indicate that transcription is more important than product. It is not yet known how many of the growing number of mammalian IncRNAs share these hallmarks. If they do, the range of IncRNA functions in the mammalian genome could be substantially enlarged.

**References and Notes**

13. See supplementary materials on Science Online.

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**Supplementary Materials**

www.sciencemag.org/cgi/content/full/338/6113/1469/DC1 Materials and Methods Figs. S1 to S12 References (21, 22)

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**A Steroid Receptor–MicroRNA Switch Regulates Life Span in Response to Signals from the Gonad**

Yidong Shen,1,2 Joshua Wollam,1,2 Daniel Magner,1,2 Oezlem Karalay,3 Adam Antebi1,2,*

Although the gonad primarily functions in procreation, it also affects animal life span. Here, we show that removal of the *Caenorhabditis elegans* germ line triggers a switch in the regulatory state of the organism to promote longevity, co-opting components involved in larval developmental timing circuits. These components include the DAF-12 steroid receptor, which is involved in the larval stage two–to–stage three (L2-L3) transition and up-regulates members of the let-7 microRNA (miRNA) family. The miRNAs target an early larval nuclear factor lin-14 and akt-1/kinase, thereby stimulating DAF-16/FOXO signaling to extend life. Our studies suggest that metazoan life span is coupled to the gonad through elements of a developmental timer.

Studies of the nematode *Caenorhabditis elegans* have shown that these animals live up to 60% longer when germline stem cells (GSCs) are eliminated from the gonad (1, 2). This longevity depends on the presence of the somatic gonad, suggesting a model wherein...
DAF-16/FOXO is also stimulated independently by decreased insulin/insulin-like growth factor (IGF) receptor (IR) signaling, because the longevity of daf-2/IR and germline-less glp-1 mutants is additive (1).

To elucidate how germline loss increases longevity, we first asked whether it affects regulation of DA signaling. When we examined mRNA levels of DA signaling components by quantitative polymerase chain reaction (PCR), we did not observe any differences between germline-less glp-1 mutants and gonad-intact wild-type (WT) animals at the third larval stage (L3). However, by L4 and day 1 of adulthood (D1), the hormone biosynthetic gene daf-36/Rieske-oxygenase was significantly up-regulated in glp-1 (Fig. 1A and fig. S1D, as well as supplementary materials and methods) but down-regulated in the wild type (Fig. 1A). Other DA-biosynthetic genes, including daf-9/CYP27A1 and dix-16/HSW, were less affected (fig. S1A) (8). Expressed mainly in the intestine, daf-36 catalyzes the first step in Δ7-DA biosynthesis, converting cholesterol to 7-dehydrocholesterol (9, 10). Accordingly, 7-dehydrocholesterol and Δ7-DA were increased four- to fivefold in glp-1 animals, as measured by gas chromatography–tandem mass spectrometry (Fig. 1, B and C). In D1 adults, daf-36 up-regulation was largely independent of daf-12 and daf-16 (fig. S1, B and C). These data suggest that a regulatory switch governs DA signaling in response to signals from the reproductive system and reveal that germline loss stimulates the DA signaling pathway.

To see whether germline loss stimulates DAF-12 transcripational activity, we focused on let-7–related microRNAs (miRNAs), mir-84 and mir-241, which are direct DAF-12 targets in larval developmental timing pathways during L2–L3 transitions (11, 12). Indeed, miRNA expression increased in glp-1 mutants by the L4 stage and peaked at three- to fourfold by D1 (Fig. 1, D and E). miRNA up-regulation was DA- and daf-12–dependent, whereas daf-16 or nhr-80, an HNF4-like nuclear receptor regulating gonadal longevity (13), had little effect (Fig. 1, F and G, and fig. S2F). Consistently, mir-84p::gfp and mir-241p::gfp promoter constructs also expressed transcriptional up-regulation in glp-1 mutants, particularly in epidermal seam cells (mir-84) and intestinal cells (mir-241), revealing complex tissue-specific regulation (Fig. 1E and fig. S2E). Other members of this miRNA family, let-7 and mir-48, were also up-regulated but showed no clear DA/DAF-12 dependence. By contrast, an unrelated miRNA, mir-228, decreased during the same time frame (fig. S2). We conclude that germline absence up-regulates DA signaling, accompanied by transcriptional activation of let-7 family members, including the DAF-12 target genes mir-84 and mir-241.

To test whether the miRNAs function in the gonadal longevity pathway, we removed GSCs

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1Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Strasse 9b, D-50931 Cologne, Germany. 2Department of Molecular and Cellular Biology, Huffington Center on Aging, Baylor College of Medicine, Houston, TX 77030, USA. 3Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, D-50674 Cologne, Germany.

*To whom correspondence should be addressed. E-mail: antebi@age.mpg.de

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Fig. 1. Ablation of the germ line up-regulates DA/DAF-12 signaling. (A) Time course of daf-36 mRNA levels in WT and germline-less animals (glp-1). Error bars denote SEM. (B and C) 7-dehydrocholesterol (B) and Δ7-DA (C) are elevated in germline-less animals. t test; **P < 0.01, ***P < 0.001. (D) Time course of mir-84 and mir-241 levels in WT and glp-1 animals. (E) In glp-1 animals, mir-84p::gfp and mir-241p::gfp expression are up-regulated in seam cells (arrowheads) and intestine (arrows), respectively. (F and G) Germline ablation results in up-regulation of mir-84 and mir-241 in a DAF-12/DAF-12–dependent manner. Strains were grown on plates supplemented with ethanol (EtOH) or Δ7-dafachronic acid (DA). Analysis of variance (ANOVA) versus glp-1 EtOH; ns, nonsignificant. *P < 0.05, **P < 0.01, ***P < 0.001.
by laser microsurgery in WT and mir-84;mir-241 mutants. As expected, life span was extended in germline-ablated WT animals compared with mock-ablated controls. Whereas gonad-intact mir-84;mir-241 controls resembled WT animals, life-span extension was abolished in germline-ablated mir-84;mir-241 double mutants (Fig. 2A). Similarly, miRNA loss suppressed longevity and stress resistance in glp-1 mutants (fig. S3 and table S1). By contrast, mir-84;mir-241 mutation had little effect on longevity caused by reduced mitochondrial function (cco-1RNAi; RNAi, RNA interference) or IR signaling (daf-2RNAi) (Fig. 2B). mir-84 and mir-241 transgenes driven by endogenous promoters restored stress resistance and longevity in mir-84;mir-241;glp-1 triple mutants but did not significantly extend life span in gonad-intact animals (fig. S3, B and C, and table S1). Thus, mir-84 and mir-241 are specifically required but not sufficient for life extension in the gonadal pathway. mir-48 mutants also significantly decreased glp-1 longevity, but affected WT animals as well (table S1). let-7 mutants were not analyzed because of their severe developmental defects. Therefore, we focused on mir-84 and mir-241 for further analysis.

DAF-16/FOXO is essential for longevity in the gonadal pathway. In germline-less animals, this transcription factor accumulates in intestinal nuclei, where it regulates genes important for life span extension (1, 6). Both DAF-12 and DAF-36 promote DAF-16 nuclear localization (4, 6); DAF-12 and DAF-16 share transcriptional responsibilities for longevity (6). To investigate whether the miRNAs interact with DAF-16, we analyzed life span upon daf-16RNAi. If miRNA deficiency reduces glp-1 longevity by a mechanism independent of daf-16, then mir-84;mir-241;glp-1;daf-16RNAi animals should live even shorter...
MicroRNAs down-regulate gene expression by binding to the 3′ untranscribed region (UTR) of target mRNAs and reducing stability and translation. To explore targets of mir-84;mir-241 and how they stimulate DAF-16/FOXO activity, we searched for DAF-16/FOXO inhibitors with potential mir-84;mir-241 binding sites in their 3′UTRs, using the bioinformatic algorithm mirWIP (16). Among predicted targets were components of IR signaling, including the PDK/AKT kinase cascade, which inhibits DAF-16/FOXO (17).

Consistent with miRNA-mediated inhibition, mir-84 and mir-241 significantly repressed luciferase reporters containing the 3′UTRs of akt-1 and pdk-1 in cell culture (fig. S7, A, G, and H). In C. elegans, akt-1-3′UTR dual reporter (DR) constructs were up-regulated in mir-84;mir-241 double mutants relative to WT animals, whereas an unc-1-3′UTR-DR control reporter remained unchanged (Fig. 3, A and B, and fig. S7C), indicating an inhibition of the akt-1 3′UTR by mir-84 and mir-241 in vivo. Moreover, the akt-1-3′UTR-DR construct was down-regulated 40 to 50% in glp-1 animals as compared with WT animals (Fig. 3, C and D). In contrast, a pdk-1-3′UTR-DR construct was not down-regulated in glp-1 animals (fig. S7I). These results indicate that mir-84;mir-241 down-regulates akt-1 through its 3′UTR, in response to gonadal signals.

When we examined functional interactions between akt-1 and the miRNAs, we observed that akt-IRNAi extended the life span of WT and glp-1 mutants by 27 and 23%, respectively (Fig. 3E and table S1), consistent with previous results (18). Notably, akt-IRNAi enhanced longevity in mir-84;mir-241;glp-1 triple mutants to the same absolute extent as in glp-1 (Fig. 3E), suggesting that akt-1 knockout bypasses the requirement for the miRNAs. Correlatively, akt-IRNAi restored expression of daf-16 target genes sod-3 and lipl-4 in mir-84;mir-241;glp-1 animals (Fig. 4F and fig. S7F). These results argue that akt-1 acts in the gonadal pathway, where the miRNAs normally antagonize akt-1 to promote longevity, as well as independently through canonical insulin/IGF signaling (18). Because daf-2/IR and gonadal longevity are additive, akt-1 could be regulated by inputs other than daf-2 upon germline ablation and may serve as a general regulator of daf-16/FOXO.

The let-7 family of miRNAs targets several genes in the heterochronic pathway, a circuit that controls larval developmental timing (19). These include genes encoding the zinc-finger protein HBL-1/hunchback and the ring-finger protein LIN-41/trim71. By mirWIP, other heterochronic genes, including those encoding nuclear protein LIN-14 and the let-7 binding protein LIN-28 are
predicted targets. If these genes are miRNA targets in the gonadal pathway, then their downstream regulation should restore longevity to mir-84/mir-241/glpr-1 triple mutants. RNAi treatment from L4 onwards revealed that only lin-14RNAi restored life-span extension to the triple mutants (Fig. 4A and table S1).

lin-14 is an intriguing candidate because Slack had previously shown that lin-14 loss of function extends life span in a daf-16/FOXO-dependent manner, and lin-14 gain-of-function mutations shorten life span (20). During development lin-14 governs L1-L2 transitions, but its context in aging is unclear. We found that lin-14RNAi extended life span in WT animals as reported, but it did not further extend the life of glp-1 mutants (Fig. 4A). To examine its relationship with daf-16/FOXO, we tested whether lin-14 knockdown influenced daf-16 target gene expression. Similar to aging experiments in which longevity was restored, lin-14RNAi also significantly restored daf-16 expression of sod-3 and lpl-4 to mir-84/mir-241/glpr-1 (Fig. 4F and fig. S7F). Altogether, these observations suggest that the miRNAs down-regulate lin-14 and promote longevity via daf-16.

To test this hypothesis, we examined regulation of lin-14 by mir-84/mir-241. As above, a luciferase reporter with the lin-14-3UTR was down-regulated by miRNAs (fig. S7B). Similarly, a lin-14-3UTR-DR construct was up-regulated in mir-84/mir-241 double mutants relative to WT, whereas the unc-1-3UTR-DR controls were unchanged (Fig. 4, B and C). Consistent with a role in the gonadal pathway, the lin-14-3UTR-DR construct and full-length lin-14::gfp were down-regulated in intestinal nuclei of germline-less animals relative to gonad-intact controls (Fig. 4, D and E, and fig. S7, D and E). Collectively, these results reveal that lin-14, a core component of the developmental clock, functions in the gonadal longevity circuit, where it is down-regulated by miRNAs upon germline removal.

In this work, we show that components of an early life developmental timing switch (i.e., the steroid receptor DAF-12; its ligands; its target miRNAs of the let-7 family; and LIN-14, the miRNAs’ target) are used to regulate adult life span in response to signals from the gonad. We propose a model in which they work as part of a hormone-regulated switch between reproductive and survival modes at larval to adult stage commitments (fig. S8). When GSC proliferation is prevented, unknown signals up-regulate daf-36 and DA production by the L4/young adult stage, subsequently activating DAF-12 and its miRNA targets, mir-84 and mir-241. In turn, these miRNAs down-regulate akt-1, lin-14, and possibly other targets, which stimulate DAF-16/FOXO transcriptional activity, extending survival and life span. Because miRNA deletion does not fully abolish DAF-16 activity, other signals from either gonad or DAF-12 may also prompt gonadal longevity. Conversely, when GSC proliferation ensues, DA signaling is down-regulated, miRNA expression is low, and lin-14 and akt-1 expression are high, resulting in normal life span. This switch could provide a critical link between development and longevity, serving as a checkpoint monitoring the state of the germ line. For example, germline absence could mimic endogenous stress signals induced by germline quiescence or proliferative arrest in response to nutrient deprivation, infection, or damage. As components of a developmental timer, the hormone-miRNA axis could ensure coordinate metabolism, maturation, and the relative timing of events between the reproductive system and the soma, with ultimate effects on life span. It will be interesting to dissect the interaction of other miRNAs implicated in longevity with this axis (20, 21). Furthermore, these findings extend the role of let-7 family members beyond developmental timing and differentiation to the regulation of insulin/IGF signaling and metabolism, similar to recent studies in mammals (22). Because let-7 family members and other components of this circuitry are evolutionarily conserved, it will be interesting to see if similar pathways affect longevity in vertebrates.

References and Notes

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Supplementary Materials
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Materials and Methods
Figs. S1 to S8
Tables S1 to S3
References (23–28)

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Hox Genes Regulate Digit Patterning by Controlling the Wavelength of a Turing-Type Mechanism

Rushikesh Sheth,1* Luciano Marcon,2,3* M. Félix Bastida,1,4 Marisa Junco,1 Laura Quintana,2,3 Randall Dahm,2 Marie Knita,4 James Sharpe,2,4,7 ‡ Maria A. Ros1‡

The formation of repetitive structures (such as stripes) in nature is often consistent with a reaction-diffusion mechanism, or Turing model, of self-organizing systems. We used mouse genetics to analyze how digit patterning (an iterative digit/ nondigit pattern) is generated. We showed that the progressive reduction in Hoxa13 and Hoxd11/Hoxd13 genes (hereafter referred to as distal Hox genes) from the Gli3-null background results in progressively more severe polydactyly, displaying thinner and densely packed digits. Combined with computer modeling, our results argue for a Turing-type mechanism underlying digit patterning, in which the dose of distal Hox genes modulates the digit period or wavelength. The phenotypic similarity with fish-fin endoskeleton patterns suggests that the pentadactyl state has been achieved through modification of an ancestral Turing-type mechanism.

Digit patterning has commonly been interpreted in the context of a morphogen gradient model (1, 2). The proposed morphogen Sonic hedgehog (Shh) emanates from the zone of polarizing activity (a cluster of mesodermal cells in the posterior border of