In key C. elegans adult tissues, the lin-4 miRNA may act to suppress the translation of lin-14, preventing lin-14 from affecting the transcription of a yet unidentified factor that regulates or interacts with thedaf-2 insulin/IGF-1 pathway. By demonstrating that lin-4 and lin-14, two key temporal regulators of development, also influence the rate of aging, we provide support for the theory that life span is affected by an innate, programmed timing mechanism. However, our data are also consistent with an alternative theory of aging, antagonistic pleiotropy, which posits that genes with primary roles in development can later secondarily influence life span (27). miRNAs are important regulators of development, apoptosis, and metabolism (28–31), and our work demonstrates that a miRNA can regulate aging, possibly through the insulin-like signaling pathway. It is possible that the mammalian lin-4 miRNA homologs, the miR-125 family, may regulate processes responsible for life-span determination in vertebrates.

References and Notes

Vertebrate regeneration is of scientific and medical interest. Although acute tissue regeneration in humans is limited, other vertebrates possess extraordinary regenerative capabilities. Zebrafish are amenable to genetic analyses and regenerate an impressive array of structures, including spinal cord, optic nerve, heart, and fins (1–3). Zebrafish fin regeneration is marked by five stages: regeneration epithelialization, formation of regeneration epithelium, and does not form blastema. This mutation has no impact on embryonic survival. Dob results from an fgg20a null mutation, Y148S. Fgg20a is expressed during initiation of fin regeneration at the epithelial-mesenchymal boundary and later overlaps with the blastema marker msxβ. Thus, fgg20a has a regeneration-specific requirement, initiating fin regeneration, and controlling blastema formation.

f gg 2 0 Is Essential for Initiating Zebrafish Fin Regeneration
Geoffrey G. Whitehead, Shinji Makino, Ching-Ling Lien, Mark T. Keating*

Epimorphic regeneration requires the presence or creation of pluripotent cells capable of reproducing lost organs. Zebrafish fin regeneration is mediated by the creation of blastema cells. Here, we characterize the deed of blastema (dob) mutant that fails fin regeneration during initial steps, forms abnormal regeneration epithelium, and does not form blastema. This mutation has no impact on embryonic survival. Dob results from an fgg20a null mutation, Y148S. Fgg20a is expressed during initiation of fin regeneration at the epithelial-mesenchymal boundary and later overlaps with the blastema marker msxβ. Thus, fgg20a has a regeneration-specific requirement, initiating fin regeneration, and controlling blastema formation.

V e r t e b r a t e r e g e n e r a t i o n is of scientific and medical interest. Although acute tissue regeneration in humans is limited, other vertebrates possess extraordinary regenerative capabilities. Zebrafish are amenable to genetic analyses and regenerate an impressive array of structures, including spinal cord, optic nerve, heart, and fins (1–3). Zebrafish fin regeneration is marked by five stages: regeneration epithelialization, mesenchymal disorganization, blastema formation, regenerative outgrowth, and termination. Although genetic analyses have enhanced our understanding of fin regeneration (1, 4–6), the specific signaling factor(s) that initiate regeneration and blastema formation are unknown.

To discover genes that initiate regeneration, we treated zebrafish with N-ethyl-N-nitrosourea (ENU) and screened adults for mutants (1, 4–6). We looked for temperature-sensitive (ts) effects on regeneration, because many genes involved in regeneration also function during embryogenesis (1, 4–7). The do b mutant displayed an early, genetically recessive regeneration block at 2 days post- amputation (dpa) at 33°C (Fig. 1). Mutant fins were covered only by epithelia, whereas wild-type fins grew beyond the amputation plane and later fully regenerated (Fig. 1). Most (72%, 34/47) mutants showed an identical regeneration defect at 25°C (fig. S2A). dob also failed to regenerate pectoral, dorsal, and anal fins. Thus, a regenerative block in do b is observed at both temperatures.

We expected that dob would also disrupt embryogenesis (1, 4–6). However, at 33°C, do b viability was comparable with wild type (fig. S1A). Half (23/46) of dob adults developed asymmetric caudal fin lobes when heat-shocked as embryos, yet all wild type (41/41) developed symmetric fin lobes (fig. S1B). The total size of wild-type and dob-causal fins was comparable (fig. S1B). Therefore, there appears to be an incompletely penetrant ts patterning defect in dob (8). Survival of dob adults at 33°C was also comparable to wild type (fig. S1C). These data suggest a regeneration-specific requirement for dob.

To determine the cellular nature of dob regenerative failure, we examined histology of regenerates at 33°C. The first stage of regeneration, formation of regeneration epithelium, appeared abnormal. At 6 and 12 hours post-amputation (hpa), dob regenerates demonstrated a thickened regeneration epithelium (Fig. 2A). Epithelial proliferation levels in dob at 6 and 12 hpa were similar to wild type (fig. S2A). Therefore, thickened regeneration epithelium likely results from aberrant epithelial migration (9, 10).

To determine whether dob resulted from a primary defect in wound healing, we performed a longitudinal incision along the caudal fin and allowed healing at 33°C. The wild-type response to this injury is nonregenerative, as the wound is covered by epithelium and leaves a slit down the fin. We found no difference in the timing, histochemistry, or bromodeoxyuridine (BrdU) immunohistochemistry of wound-healing between wild type and dob (fig. S3B). The possibility remains that the dob mutant may have a subtle defect in wound-healing not identified by our observations.

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To further characterize the dob defect in regeneration epithelialization, we performed in situ hybridization experiments. The wild-type regeneration epithelium at 24 hpa is molecularly and histochemically distinct. Specifically, the basal epithelium in wild-type regenerates consists of ordered linear cuboidal epithelial cells (11). In dob, the basal epithelium lacked the distinctive cuboidal shape and was nonlinear. Lef1, a transcription factor downstream of Wnt (12), and sparc, a matricellular protein (13), both demarcate regeneration basal epithelium. Lef1 and sparc in situ hybridization in dob revealed absent basal epithelial expression (Fig. 2B). Thus, normal formation of a basal regeneration epithelium appears essential for fin regeneration.

At 18 hpa in wild-type regenerates, disorganized mesenchymal cells beneath the amputation plane are considered evidence of dedifferentiation (9, 11). dob mutants did not undergo mesenchymal disorganization (Fig. 2A). In 18 hpa wild-type regenerates, hsp60 is up-regulated in mesenchymal cells destined to form blastema (6). However, dob did not express hsp60 in these cells (Fig. S2B). These data suggest a mesenchymal disorganization defect in dob.

Mesenchymal disorganization is followed by cell proliferation, migration, and blastema formation (9, 10). The blastema is a mass of undifferentiated mesenchymal cells that have proliferated beyond the amputation plane to drive fin regrowth. At 36 hpa, wild-type regenerates show proper blastema formation; however, dob is devoid of blastema (Fig. 2A). These data indicate that dob does not initiate fin regeneration and fails to form regeneration epithelium and blastema.

To determine the effect of dob on blastema formation, we performed in situ hybridization experiments. In 24 hpa wild-type fins, msxb marks rudimentary blastema cells, the mesenchyme distal to the amputation plane. At 72 hpa, during regenerative outgrowth, msxb marks distal blastema (7, 9). No msxb expression was present in dob at 24 hpa during blastema formation (Fig. 2B). Faint msxb expression was present in dob at 72 hpa at the central tip of mesenchyme (fig. S2C). These msxb-positive cells may represent a later, inadequate attempt at blastema formation. These data demonstrate that dob lacks early msxb expression and does not form blastema.

During blastema formation, mesenchymal cells reenter the cell cycle and begin to proliferate (9, 10). These cells migrate toward regeneration epidermis and form the rudimentary blastema. To further define the mechanism of the dob regenerative defect, we examined DNA replication, through BrdU labeling. At 24 hpa, dob mesenchymal proliferation levels were one-third of wild type, and epithelial proliferation was slightly lower (Fig. 2C). These data indicate that dob fails to form a blastema through an early defect in mesenchymal proliferation.

To identify the dob gene, we raised 2027 zebrafish from dob−/− × dob−/− mapping crosses to adulthood at 25°C, scored for regenerative defects at 33°C, and genotyped these animals (1, 4–6). Two markers, bef7 and tof24, flanked the 0.2 centimorgan (cM) dob critical region on chromosome 1. fgf20a was the only transcript within this region, genetically excluding neighboring transcripts (Fig. 3A). Syntenic multicontig alignments demonstrated that no transcripts were located between efha2 and fgf20a in human or fugu databases.

To identify the dob mutation, we performed DNA sequence analysis of fgf20a. We discovered one missense mutation, an adenine-443 to cytosine (A443C) transversion, in the fgf20a gene of dob that converted tyrosine-148 to serine (Y148S) (Fig. 3B). We genotyped 140 dob mutants, 30 dob heterozygotes, and 20 wild-type controls and verified that the A443C transversion cosegregated with the dob phenotype. Dob was isolated in the SJD background, and DNA sequence of fgf20a in wild-type SJD revealed no mutation. Therefore, the A443C transversion in dob was caused by ENU mutagenesis. This point mutation was not found among five commonly used laboratory strains, indicating that A443C is not a polymorphism. These data indicate that the dob phenotype results from fgf20a Y148S.

Fgf20 is a newly identified member of the Fgf family. Fgf20 is overexpressed in cancer cell lines, promotes proliferation and differentiation of myocardial cells, and enhances survival of dopaminergic neurons in the adult brain, functions consistent with a role in regeneration (14–16). Y148 exists in the highly
Fig. 3. fgf20a Y148S missense mutation causes dob. (A) Genetic map of dob on chromosome 1. Refined linkage analysis mapped dob to the 0.2 cM region between bef7 and tof24. The only gene between flanking recombinant markers is fgf20a. Numbers above linear map quantify recombination events between dob and linked markers from 2027 meioses. bef7, fe12, mt254, tof24, and et6 are polymorphic genetic markers between AB and SJD strains identified by random DNA amplification and sequencing within the dob critical region. (B) DNA sequence chromatograms of wild-type, dob–/–, and dob+/– fish. A443C transversion leads to Y148S amino acid substitution. (C) Tyr-148 is conserved across vertebrates and among most zebrafish Fgfs. (D) Fgf secondary β-trefoil structure. Tyr-148 is located in the β9 strand of Fgf20a. (E) Phenotypic classes and frequencies (%) obtained after injection of wild-type fgf20a, Y148S fgf20a, wild-type fgf3, or Y148C fgf3 mRNAs (10 ng/µl) into wild-type embryos. Y148S fgf20a had no effect on the embryo, suggesting loss of function. Wt, normal; p1, head reduction, loss of tail; p2, lysis.

Fig. 4. fgf20a expression localizes to epithelial-mesenchymal boundary during initiation of fin regeneration. (A) Whole-mount in situ hybridization and sections showing fgf20a expression. During initiation of fin regeneration (6 to 12 hpa), fgf20a expression is localized to mesenchymal cells directly underneath the regeneration epithelium. During early blastema formation (18 hpa), fgf20a is confined to the blastema. (B) During blastema formation (24 hpa), fgf20a and msxb colocalize in blastema cells. fgf20a and msxb expression domains overlap at 72 hpa, when fgf20a is concentrated at the distal tip of msxb-positive distal blastema. Violet stain indicated by red arrow marks expression. Scale bars, 100 µm.

To determine the effect of Y148S on the activity of Fgf20a protein, we carried out overexpression studies in zebrafish embryos. Injection of wild-type fgf mRNA leads to dorsalization of the embryo and death (18). Injected fgf mRNA Y148S mRNA failed to recapitulate this phenotype (Fig. 3E). Similar results have been demonstrated for the null fgf21142 allele, a Y148C mutation, the same tyrosine mutated in dob (18). These data indicate that Y148S is likely a null mutation and that Y148 is crucial for Fgf function.

To ensure that fgf20a Y148S is responsible for the dob phenotype, we injected wild-type and dob embryos with wild-type fgf20a mRNA. We found that 87.8% of wild-type embryos showed dorsalization and lethality, compared with 53.5% of dob embryos (P = 0.02) (table S1). These data support the view that the dob phenotype results from reduced fgf20a and that fgf20a Y148S causes dob.

We define the timing and pattern of fgf20a expression during fin regeneration, we performed reverse transcriptase polymerase chain reaction and in situ hybridization experiments. fgf20a was expressed as early as 1 hpa. Expression peaked at 6 hpa, gradually declined, peaked again at 24 hpa, then declined (fig. S2D). During initiation of fin regeneration (6 to 12 hpa), fgf20a expression was localized to mesenchyme at the epithelial-mesenchymal boundary (Fig. 4A). fgf20a was later expressed in blastema during early blastema formation (18 hpa) (Fig. 4A). These data indicate that fgf20a is expressed in key regeneration cells during initiation of fin regeneration, consistent with the dob phenotype.

We further characterized the expression of fgf20a during fin regeneration and compared fgf20a and msxb expression. At 24 hpa, msxb marks rudimentary blastema cells. fgf20a expression colocalized with msxb in early blastema cells (Fig. 4B). During regenerative outgrowth, at 72 hpa, msxb expression marks stem cell–like distal blastema cells (6, 9). fgf20a expression was restricted to a subset of msxb-positive distal blastema cells (Fig. 4B). These data indicate that fgf20a and msxb expression overlap during blastema formation and regenerative outgrowth.

We conclude that the dob phenotype is caused by a Y148S mutation in Fgf20a. Data implicating fgf20a in dob include (i) genetic linkage of dob to a 70-kb critical interval on chromosome 1, the presence of fgf20a in this interval, and the absence of other transcripts; (ii) the presence of Fgf20a missense mutation (Y148S) in a completely conserved amino acid linked with the dob phenotype (P = 4.22 × 10−42, Fisher’s Exact test); (iii) the absence of Y148S in five wild-type strains, indicating that this variant is not a polymorphism; (iv) in situ hybridization showing that fgf20a is expressed in mesenchymal cells adjacent to regeneration epithelium as early as

conserved Fgf-core domain in the β9 strand of the 12-strand β-trefoil, adjacent to residues (WYN) (Fig. 3, C and D) that make essential Fgf receptor contacts (17). Y148 is invariant across vertebrate species of Fgf20 and most zebrafish Fgfs (Fig. 3C). Thus, Y148 is evolutionarily conserved among species and within the Fgf family.
Protein Synthesis upon Acute Nutrient Restriction Relies on Proteasome Function

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The mechanisms that protect mammalian cells against amino acid deprivation are only partially understood. We found that during an acute decrease in external amino acid supply, before up-regulation of the autophagosomal-lysosomal pathway, efficient translation was ensured by proteasomal protein degradation. Amino acids for the synthesis of new proteins were supplied by the degradation of preexisting proteins, whereas nascent and newly formed polypeptides remained largely protected from proteolysis. Proteasome inhibition during nutrient deprivation caused rapid amino acid depletion and marked impairment of translation. Thus, the proteasome plays a crucial role in cell survival after acute disruption of amino acid supply.

Protein biosynthesis in mammalian cells relies on the continuous uptake of essential amino acids from the environment. Acute amino acid restriction can occur in several physiological and pathophysiological conditions, such as after disruption of the trans-placental nutrient supply in neonates or during organ ischemia. Up-regulation of the autophagosomal-lysosomal pathway is known to provide free amino acids for protein synthesis under these nutrient stress conditions through the bulk degradation of cytoplasmic proteins and organelles (1, 2). However, this adaptation requires hours to become fully effective (2, 3), suggesting the existence of constitutive mechanisms that protect cells during short-term fluctuations in amino acid supply. Moreover, certain organs, such as the brain, are inefficient in up-regulating autophagy (3). Nonlysosomal degradation by means of the proteasomal system is known to be induced during long-term fasting (4), but the role of the proteasome in maintaining the intracellular amino acid pool during acute nutrient deprivation has remained unexplored. The proteasome, a ~2.5-MD protein complex acting together with ubiquitin and ubiquitin-processing enzymes, is responsible for most cytosolic protein degradation under normal nutrient conditions and has a variety of essential functions in cell regulation and protein quality control (5, 6).

A constitutive function of the proteasome in buffering fluctuations in external amino acid availability would be consistent with a recent report that 30% or more of newly formed proteins are proteasomally degraded immediately upon synthesis (7). To explore a possible role of the proteasome in supplying amino acids for translation, we established conditions to measure the immediate effects of proteasome inhibition in human HeLa cells. A rapidly degraded ubiquitin–enhanced green fluorescent protein fusion protein (Ub-EGFP) served as a reporter of proteasome activity (8, 9). Ub-EGFP accumulated to a low steady-state level in transiently transfected cells, reflecting the equilibrium between its synthesis and degradation. As expected, upon inhibition of protein synthesis with cycloheximide (CHX), Ub-EGFP was degraded within minutes (Fig. 1A). In contrast, the addition of the proteasome inhibitor MG132 caused the virtually immediate accumulation of Ub-EGFP (Fig. 1A). To determine whether proteasome inhibition was complete, we analyzed the combined effect of MG132 and CHX. Inhibition of translation by CHX is known to be very rapid and efficient (10, 11). Thus, if MG132 were to block proteasome function only partially, the arrest of translation would lead to a decrease in Ub-EGFP level due to degradation. The simultaneous addition of CHX and MG132 instantaneously stabilized the Ub-EGFP reporter (Fig. 1A). Similar observations were made with the proteasome inhibitors clasto-lactacystin-β-lactone and epoxomicin (11). Thus, under the conditions chosen proteasome inhibition was immediate and essentially complete.

The effect of proteasome inhibition on translation was analyzed under conditions of acute amino acid restriction. The concentrations of the essential amino acids—leucine, phenylalanine, or methionine—were maintained in the range of normal adult plasma levels (12) or were reduced individually 100-fold to create insufficiency in external supply (13). Newly synthesized proteins were labeled with [35S]-methionine ([35S]-Met), followed by cell lysis in SDS and precipitation of proteins with trichloroacetic acid (TCA). Proteasome inhibition with MG132, clasto-lactacystin-β-lactone or epoxomicin markedly impaired translation within 5 to 10 min, but only when cells were incubated in medium deficient in at least one essential amino acid (leucine or phenylalanine) (Fig. 1, B to D, and fig. S1, A and B). Similar results were obtained when cells were incubated in methionine-deficient medium with [3H]-leucine ([3H]-Leu) as the tracer.
Editor's Summary

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