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MAB-10/NAB acts with LIN-29/EGR to regulate terminal differentiation and the transition from larva to adult in *C. elegans*

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SUMMARY

In *Caenorhabditis elegans*, a well-defined pathway of heterochronic genes ensures the proper timing of stage-specific developmental events. During the final larval stage, an upregulation of the *let-7* microRNA indirectly activates the terminal differentiation factor and central regulator of the larval-to-adult transition, LIN-29, via the downregulation of the *let-7* target genes *lin-41* and *hbl-1*. Here, we identify a new heterochronic gene, *mab-10*, and show that *mab-10* encodes a NAB (NGFI-A-binding protein) transcriptional co-factor. MAB-10 acts with LIN-29 to control the expression of genes required to regulate a subset of differentiation events during the larval-to-adult transition, and we show that the NAB-interaction domain of LIN-29 is conserved in Kruppel-family EGR (early growth response) proteins. In mammals, EGR proteins control the differentiation of multiple cell lineages, and EGR-1 acts with NAB proteins to initiate menarche by regulating the transcription of the luteinizing hormone β subunit. Genome-wide association studies of humans and various studies of mouse recently have implicated the mammalian homologs of the *C. elegans* heterochronic gene *lin-28* in regulating cellular differentiation and the timing of menarche. Our work suggests that human homologs of multiple *C. elegans* heterochronic genes might act in an evolutionarily conserved pathway to promote cellular differentiation and the onset of puberty.

KEY WORDS: Heterochronic, Molting, Terminal differentiation

INTRODUCTION

C. elegans heterochronic genes have been characterized based in large part upon their affects in regulating the biology of lateral hypodermal cells known as seam cells. Seam cells undergo a stem-cell-like pattern of asymmetric cell division during each larval stage (Sulston and Horvitz, 1977). At the end of each larval stage, the hypoderm generates a new cuticle and the animal sheds its old cuticle. After the final (L4) larval stage, the hypoderm undergoes a process of terminal differentiation that comprises four events: (1) synthesis of the adult-specific cuticle; (2) exit from the molting cycle; (3) seam cell fusion; and (4) seam cell exit from the cell cycle. Terminal differentiation is initiated via the downregulation of the Hunchback/Ikaros homolog *hbl-1* and the TRIM-NHL gene *lin-41* by the *let-7* family of miRNAs. This downregulation triggers the activity of the Kruppel family zinc-finger protein LIN-29, which promotes all four aspects of terminal differentiation (Fig. 1B) (Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003). Seam cell fusion, exit from the cell cycle, and synthesis of the adult-specific cuticle occur during the L4 stage, but despite the presence of LIN-29 activity, exit from the molting cycle does not occur until after the animal becomes an adult. This observation suggests that exit from the molting cycle is not solely dependent on the presence of LIN-29 activity and that there might be other factors that work with LIN-29 to ensure its proper regulation.

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MATERIALS AND METHODS

Strains and genetics

C. elegans was grown as described previously (Brenner, 1974) and maintained at 20°C unless otherwise noted. N2 was the wild-type strain. The mutations used in this study were: LGI, *smg-1(e1228)* (Hodgkin et al., 1989), *lin-28(n719)* (Ambros and Horvitz, 1984), *lin-41(n2914)* (Slack et al., 2000); LGII, *dpy-10(e128)* (Brenner, 1974), *mab-10(e1248)* (Hodgkin, 1983), *mab-10(n5117)*, *mab-10(n5118)*, *mab-10(tm2497)*, *lin-29(n546)* (Ambros and Horvitz, 1984), *lin-29(n836)* (Papp et al., 1991) and *rol-1(e91)* (Brenner, 1974); LGIV, *him-8(e1489)* (Hodgkin et al., 1979); LGV, *him-5(e1467)* (Hodgkin et al., 1979).

Information about *tm2497* (kindly provided by S. Mitani, Tokyo Women's Medical University, Japan) can be found at www.wormbase.org. The following balancer chromosomes were used: *hT2 [qIs48]* LGI; LGIII, *mIn1 [mIs14]* LGII and *mnC1* LGII (Edgley et al., 2006).

Screen for males with an extra cuticle

The F2 progeny of mutagenized *him-8(e1489)* hermaphrodites were screened clonally using a dissecting microscope for adult males with an extra cuticle. We screened about 4500 genomes and isolated two mutants (*n5117* and *n5118*), both of which failed to complement *mab-10(e1248)*.

Phenotypic analyses

Cuticles were observed using a Zeiss Axioskop 2 with Nomarski optics. Seam cell fusion was assayed in early L4 and young adult animals using the *ajm-1::gfp (wIs78)* (Koh and Rothman, 2001) reporter. Adult molting was assayed by picking L4 males to plates and scoring 24 hours later for the presence of an extra cuticle. Synchronized wild-type and *mab-10(e1248)* males and hermaphrodites were grown until the *mab-10* mutants had synthesized an extra cuticle. These animals were then fixed and electron microscopic analysis was performed as described previously. Seam cell divisions were followed using either Nomarski microscopy or fluorescence microscopy to observe *scm::gfp (wIs78)* (Koh and Rothman, 2001) or *col-19::gfp (maIs105)*, as noted in figure legends. To assay adult seam cells, we picked late L4 animals to fresh plates and scored 24 hours later.

Plasmids

pDH04 contains about 9 kb of the *mab-10* genomic locus on LG II from 10,525,019 to 10,534,380 with *gfp* inserted in place of the stop codon. pDH36 contains about 18 kb of the *lin-29* genomic locus on LG II from 11,917,276 to 11,936,077 with *mCherry* inserted in place of the stop codon. *mab-10* cDNA was isolated by RT-PCR from a mixed-stage RNA preparation and cloned into the PCR8GW Gateway entry vector creating pDH08. pDH23 contains the *mab-10* cDNA fused downstream of *gst* and was created by the Gateway LR reaction between pDH08 and pDestGST2TK. Full-length *lin-29* cDNA was amplified from the plasmid AAC37255 (Open Biosystems) and cloned into the PCR8GW entry vector, creating pDH06. pDH24 contains the *lin-29* cDNA fused downstream of 6XHis tag and was created by the Gateway LR reaction between pDH06 and pDest17. All plasmids containing *lin-29* deletion variants were generated by site-directed mutagenesis of pDH24.

Oligos

The oligos used were: pDH06 (5′-atggatcaaactgttctagattcggc-3′ and 5′-ttaataggaatgatttttcatattat-3′; pDH08 (5′-atgtcatcatcgtcgtcgtcgtcgtta-3′ and 5′-tcaagattccgggagctcacccttcatttttcgattatcgccgcccat-3′; pDH25 (LIN-29 Del 1-144) (5′-aaagcaggctccgaattcgcccttatgcagatgcgggaagcaaaaccttacaagt-3′ and 5′-acttgtaaggttttgcttcccgcatctgcataagggcgaattcggagcctgcttt-3′); pDH26 (LIN-29 Del 147-300) (5′-caaaattgctggccttcgatcgatccgctgcttcccgcatctgcatctgttgctg-3′ and 5′-cagcaacagatgcagatgcgggaagcacaaactgataagccattcaaatgtaac-3′); pDH27 (LIN-29 Del 301-459) (5′-catgacaaagcacgcggatcgatcgtaaaagggcgaattcgacccagctttc-3′ and 5′-gaaagctgggtcgaattcgcccttttacgatcgatccgcgtgctttgtcatg-3′); pDH29 (LIN-29 Del 371-389) (5′-cagtggggttatcatgttgaacccaggaatattaaagagctgactgaacgcact-3′ and 5′-agtgcgttcagtcagctctttaatattcctgggttcaacatgataaccccactg-3′); pDH30 (LIN-29 Del 390-406) (5′-acgagtacgaagaatggagaacgagcttcatcttcctcggccacggctgtcgta-3′ and 5′-tacgacagccgtggccgaggaagatgaagctcgttctccattcttcgtactcgt-3′); pDH31 (LIN-29 DEL 404-440) (5′-ctggagaacatccaacgctacaacgggcagggaggcgtgttcaacccacaatca-3′ and 5′-tgattgtgggttgaacacgcctccctgcccgttgtagcgttggatgttctccag-3′); pDH32 (LIN-29 441-459) (5′-tcctcgtcagcaggttcgtcctcaagttacccagctttcttgtacaaagtggtt-3′ and 5′-aaccactttgtacaagaaagctgggtaacttgaggacgaacctgctgacgagga-3′); pDH37 (LIN-29 Del 147-232) (5′-cagcaacagatgcagatgcgggaagcacaaactgataagccattcaaatgtaac-3′ and 5′-gttacatttgaatggcttatcagtttgtgcttcccgcatctgcatctgttgctg-3′); pDH38 (LIN-29 Del 233-300) (5′-aatctccaatctcactctcgatgccatgcggatcgatcgaaggccagcaat-3′ and 5′-attgctggccttcgatcgatccgcatggcatcgagagtgagattggagatt-3′).

Transgenics

The integrated arrays *wIs78* (Koh and Rothman, 2001), which contains *scm::gfp* and *ajm-1::gfp*, and *maIs105* (Feinbaum and Ambros, 1999), which contains *col-19::gfp*, were used to assay seam cell fusion and seam cell number. The *mab-10::gfp* array (*nEx1655*) was formed by the co-injection of pDH04 at 35 ng/μl and pSN359 [*pgp-12::gfp* (Zhao et al., 2005)] at 40 ng/μl. The *lin-29::mCherry* array (*nEx1681*) was formed by the injection of PCR product (50 ng/μl) containing LG II sequence from 11917298 to 11927996 from template pDH36 with *ttx-3::gfp* (Hobert et al., 1997) at 40 ng/μl. *nEx1681* was integrated into the genome using γ irradiation to form *nIs408.*

GST pull-down assay

GST pull-downs were performed as previously described (Reddien and Horvitz, 2000) and quantified using a Typhoon phosphorimager (GE Healthcare). In short, ^{35}S-methionine-labeled LIN-29 protein was transcribed and translated in vitro using a TNT Quick Coupled Transcription/Translation Kit (Promega) and bound to MAB-10::GST fusion protein purified from *E. coli*. Binding efficiency is expressed as the percent of wild-type binding after correcting for binding to GST alone.

Single-molecule FISH

Single-molecule FISH (fluorescence in situ hybridization) was performed based on protocols described previously (Raj et al., 2008) and at www.singlemoleculefish.com. Briefly, synchronized populations of N2 animals were grown on NGM agar plates seeded with OP50. Animals were harvested by the addition of 2-3 ml of M9 solution and subsequent collection in 15 ml conical tubes. Animals were washed twice with PBS and transferred to a 1.5 ml microfuge tube. This procedure yielded approximately 35 μl of packed animals per large NGM plate. PBS was removed and replaced with 1 ml of fixation solution (4% formaldehyde in PBS). Animals were fixed for 45 minutes and washed twice in PBS. After the second wash, the PBS was removed and replaced with 70% ethanol. Animals were kept in 70% ethanol for at least 1 night.

Hybridizations were performed essentially as described previously (Raj et al., 2008) and at www.singlemoleculefish.com. For *mab-10* and *cki-1* probes conjugated to Cy5, a probe dilution of 1/10,000 from the original stock solution was used. For *nhr-23* and *nhr-25* probes conjugated to Cy5, a probe dilution of 1/5000 from the original stock solution was used. Probes for *mab-10*, *nhr-23*, *nhr-25* and *cki-1* were designed following the guidelines specified at www.singlemoleculefish.com and purchased from Biosearch Technologies (Novato, CA).

Images were filtered using MATLAB to enhance automated transcript identification as previously reported (Raj et al., 2008). To quantify transcript numbers within the hypoderm specifically, we used MATLAB to manually define a region-of-interest around the hypoderm in three dimensions using DAPI staining as a guide. The lateral hypoderm is readily identifiable based on its morphology and position within the animal. Whole-animal hypoderm counts are the summation of five to eight individual partially overlapping stacks. Care was taken when selecting a region-of-interest to minimize double counting of transcripts within image overlaps.

RNAi

RNAi by feeding was performed as reported previously (Ashrafi et al., 2003). We used RNAi constructs from the ORFeome library for *mab-10*, *lin-29* and *cki-1*. For RNAi of *mab-10*, L4 *him-8* animals were picked individually to seeded RNAi plates containing 1 mm IPTG. Animals were moved to another RNAi plate 72 hours later. Progeny from both plates were scored for extra molts. For RNAi of *lin-29*, L4 *him-5* and L4 *wIs78*-containing animals were picked individually to seeded RNAi plates containing either 1 nm or 1 μM IPTG. Animals were transferred to new plates as described above and progeny were scored for extra molts, extra seam cell divisions, adult alae and seam cell fusion. We performed postembryonic RNAi of *cki-1* by picking multiple late L1/L2 *wIs78*-containing animals, which hatched on OP50, onto seeded RNAi plates containing 1 mm IPTG. These animals were scored for extra molts, extra seam cell divisions, adult alae and seam cell fusion.

RESULTS

A screen to identify mutants that undergo an extra molt

To identify new genes required for the terminal differentiation of the hypoderm, we performed a screen for mutants that failed to exit the molting cycle and inappropriately initiated an adult molt. Because extra molts are difficult to observe in adult hermaphrodites (as described below), we focused on the identification of mutant males. We identified two mutations, *n5117* and *n5118*, both of which failed to complement the previously isolated mutation *mab-10(e1248)*. *mab-10(e1248)* was originally identified in a screen for morphologically abnormal males (male abnormal) (Hodgkin, 1983), and *mab-10* males were later observed to undergo an extra molt (C. Link, personal communication).

mab-10 mutants are defective in a subset of events during the larval-to-adult transition

We found that *mab-10* mutants synthesized an adult cuticle appropriately at the end of the fourth larval stage but subsequently generated a second adult cuticle. All *mab-10* males underwent an extra molt, whereas not all *mab-10* hermaphrodites underwent the extra molt (Fig. 1C-F). Adult *mab-10* hermaphrodites that had begun to synthesize the second adult cuticle were typically consumed by internally hatched progeny, making the extra molt difficult to observe.

DEVELOPMENT

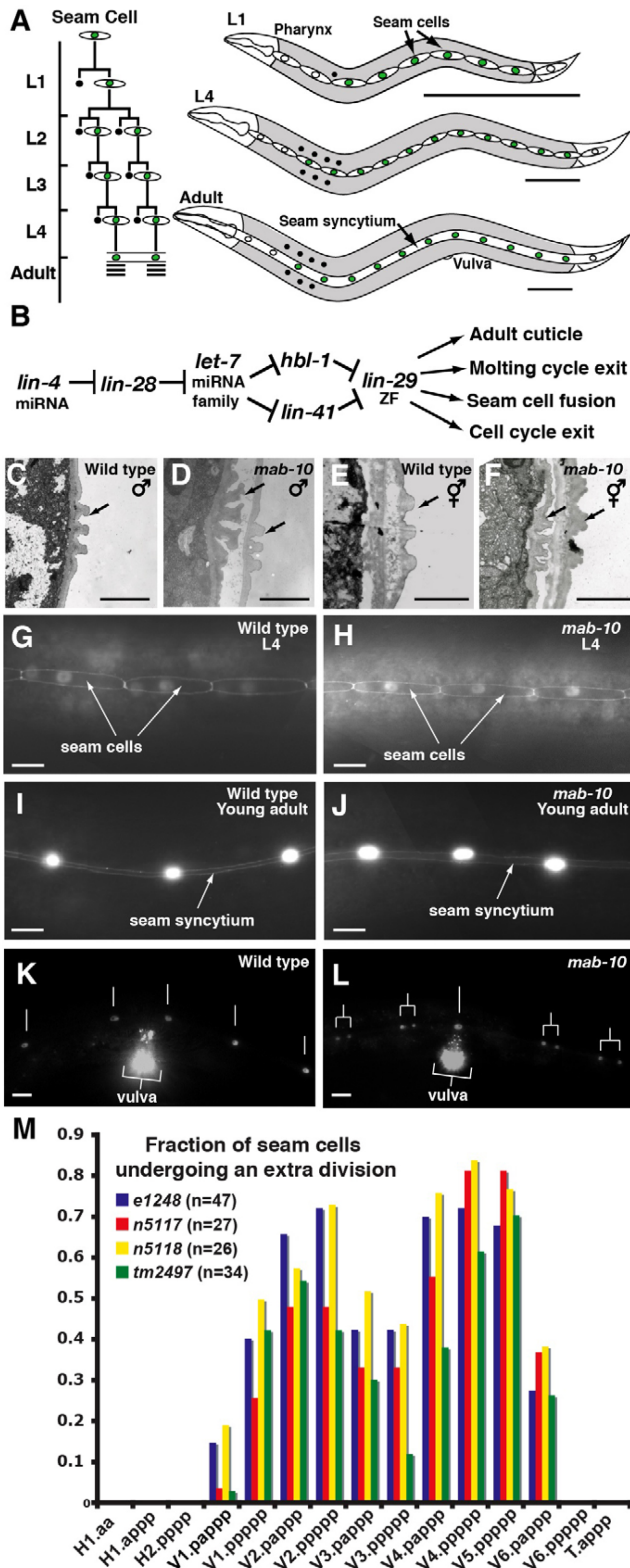

Fig. 1. ***mab-10*** **promotes hypodermal terminal differentiation.** (**A**) A wild-type seam cell lineage (V1-V4, V6) (Sulston and Horvitz, 1977). Terminal differentiation is indicated by the fusion of the seam cells (eye-shaped cells with green nuclei) and the formation of adult-specific lateral alae (three horizontal bars). To the right is a diagram of the H-, V- (green) and T-derived seam cells of L1, L4 and adult animals. Anterior daughters of a division (black) fuse with the hypodermal syncytium, while posterior daughters (green) retain stem cell characteristics. The anterior progeny from only the V1 lineage are shown (dorsal and ventral to the newly V1-derived seam cells). (**B**) Diagram of the heterochronic gene pathway that controls the four aspects of terminal hypodermal differentiation. (**C-F**) Electron micrographs of cross-sections of wild-type and *mab-10(e1248)* 18 hour adult males and hermaphrodites. Arrows indicate adult-specific lateral alae. Scale bars: 2 μm. (**G-J**) Wild-type and *mab-10(n5117)* hermaphrodite seam cell membrane and nuclei visualized using the adherens junction marker AJM-1::GFP (*wIs78*) and the seam cell reporter SCM::GFP (*wIs78*), respectively (Koh and Rothman, 2001). Scale bars: 10 μm. (**K**,**L**) Seam cell nuclei of 24 hour adult wild-type and *mab-10(n5117)* hermaphrodites visualized using the seam cell reporter SCM::GFP (*wIs78*) (Koh and Rothman, 2001). Scale bars: 10 μm. Anterior is leftwards; dorsal is upwards. (**M**) Fractions of seam cells that underwent an extra cell division in each *mab-10* mutant strain. n, number of animals assayed.

Seam cells fused normally at the larval-to-adult transition, although the fused seam occasionally appeared wider than that of the wild type (Fig. 1G-J), and the seam cell lineage during larval development was normal (data not shown). However, approximately five of the 11 V lineage-derived seam cell nuclei underwent an extra division by 24 hours after the larval-to-adult transition (Fig. 1K,L) (Table 1). The timing of the extra divisions ranged from ~14 to 20 hours post-adult, and seam cells occasionally divided twice within this interval. This result indicates that *mab-10* is required to promote seam cell exit from the cell cycle. Although present within a syncytium, the different seam cell nuclei were not equally likely to undergo an extra division (Fig. 1M).

Although the *mab-10* mutant phenotype was originally thought to be male-specific, we have shown that *mab-10* males and hermaphrodites undergo extra molts and the seam cells of mutant males and hermaphrodites continue to divide after the larval-to-adult transition. Thus, *mab-10*, like *lin-29*, is required to prevent extra molts and extra seam cell divisions and both *mab-10* and *lin-29* have a retarded heterochronic defect. However, *mab-10*, unlike *lin-29*, is not required for adult-specific cuticle synthesis or seam cell fusion.

mab-10 promotes terminal differentiation downstream of *lin-28* and *lin-41*

lin-28 and *lin-41* mutants exhibit adult characteristics one or sometimes two stages prematurely. In the case of *lin-28*, an L2-specific developmental program is skipped, resulting in a relatively complete transition to adult prematurely. Early development of *lin-41* animals proceeds normally through the L3 stage but animals then prematurely display adult characteristics, including patches of adult alae and seam cells that have exited the cell cycle.

lin-28; mab-10 animals, like *lin-28* animals, skip the L2-specific developmental program and take on adult characteristics prematurely, including the formation of complete adult alae (see Table S1 in the supplementary material); however, the seam cells continue to divide in a *mab-10*-like fashion (Table 1). This result suggests that *mab-10* acts downstream of or parallel to *lin-28* to control seam cell differentiation.

Table 1. *mab-10* is required for *C. elegans* to exit the molting cycle and for seam cell exit from the cell cycle

	% Adult alae (*n*)	% Extra molt (*n*)	% Seam cell fusion (*n*)	Seam cell number (*n*)
Wild type*	100 (34)	0 (50)	100 (34)	11.1±0.4 (34)
*mab-10(e1248)**	100 (99)	100 (50)	100 (30)	13.1±2.0 (99)[‡]
*mab-10(tm2497)**	100 (99)	100 (50)	100 (30)	15.1±2.4 (99)[‡]
*mab-10(n5118)I**	100 (93)	100 (50)	100 (30)	15.3±2.4 (93)[‡]
*mab-10(n5117)**	100 (40)	100 (50)	100 (40)	15.8±2.6 (40)[‡]
lin-28(n719)[†]	100 (15)	ND	ND	7.0±0.9 (15)
lin-28(n719); mab-10(n5117)[†]	100 (20)	ND	ND	13.0±2.3 (20)[‡]
lin-41(n2914)[†]	100 (30)	ND	ND	10.8±1.0 (30)
lin-41(n2914); mab-10(n5117)[†]	100 (34)	ND	ND	15.5±3.5 (34)[‡]
lin-29(n546)[†]	0 (50)	100 (50)	0 (50)	18.8±3.8 (22)
smg-1(e1228); lin-29(n546)[†]	100 (20)	93 (15)	100 (20)	11.1±0.5 (20)[‡]
RNAi empty vector	100 (20)	0 (19)	100 (20)	11.0±0.0 (20)
cki-1 RNAi	100 (18)	0 (25)	100 (18)	12.7±1.2 (18)[‡]

Animals were scored for the presence of complete adult alae and indicated as a percentage of sides scored. Adult males were assayed for extra molts and are indicated as a percentage of animals assayed. Animals were scored for complete seam cell fusion and are indicated as a percentage of sides scored. The V-lineage-derived seam cells of hermaphrodites were scored 24 hours after the initiation of the L4 lethargus and are indicated as total number ±s.d.
ND, not determined.
*Strains contained *wIs78* (Koh and Rothman, 2001) and *him-5(e1467ts)*.
[†]Strains contained *him-8(e1489)* and *maIs105* (Feinbaum and Ambros, 1999).
[‡]*P*<0.005 by Student's *t*-test.

Similarly, seam cells of *lin-41; mab-10* animals stopped dividing at the end of the L3 stage, but then divided again in a *mab-10*-like fashion (Table 1). Surprisingly, far fewer *lin-41; mab-10* animals displayed precocious patches of adult alae when compared with *lin-41* animals alone and the patches of those *lin-41; mab-10* animals that had precocious alae were far smaller than those in *lin-41* single mutants (see Table S1 in the supplementary material). This suppression demonstrates that *mab-10* has the capacity to promote adult alae formation despite not being required for normal adult alae formation. Although *mab-10* mutants made normal adult alae, we did observe a modest decrease in the expression of *col-19*, an adult-specific cuticle collagen (see Fig. S1A-C in the supplementary material).

These data indicate that *mab-10* likely functions downstream of or parallel to both *lin-28* and *lin-41* and that MAB-10 can promote aspects of the larval-to-adult transition beyond exit from the cell cycle and exit from the molting cycle.

mab-10 encodes a NAB transcriptional co-factor

Using a combination of SNP mapping, genomic rescue and DNA sequence determination, we determined that *mab-10* is the gene *R166.1*(NM_063867.1) (Fig. 2A; see Fig. S2 in the supplementary material) and encodes a protein similar to NAB transcriptional co-factors (Fig. 2B,C). The NAB-specific NCD1 (Nab conserved domain) domain is thought to be required for physical interactions with the R1 (repressor 1) domains of the mammalian immediate early genes EGR-1, EGR2 and EGR-3 (Russo et al., 1995), and the NCD2 domain has been shown to function both as a transcriptional activator and as a repressor (Swirnoff et al., 1998; Sevetson et al., 2000). The two strongest alleles, *mab-10(n5117)* and *mab-10(n5118)*, are both early nonsense mutations located within the NCD1 domain and are probably nulls. The weaker allele, *mab-10(e1248)*, causes a serine-to-phenylalanine amino acid substitution within the NCD2 domain (Fig. 2C).

Mammalian NAB proteins act as co-factors for EGR proteins to transcriptionally activate or repress genes required to regulate the terminal differentiation and function of several cell types: keratinocytes, chondrocytes, Schwann cells and macrophages (Swirnoff et al., 1998; Topilko et al., 1998; Sevetson et al., 2000; Le et al., 2005; Laslo et al., 2006). In the gonadotrope lineage of the anterior pituitary gland, NAB proteins act with EGR1, in conjunction with steroidogenic factor 1 (SF1), to promote transcription of the β-subunit of luteinizing hormone and the onset of puberty (Dorn et al., 1999; Wolfe and Call, 1999).

Given the similar phenotypes of *mab-10* and *lin-29* mutants, we hypothesized that MAB-10 acts with LIN-29 to promote the late-occurring aspects of terminal differentiation during the larval-to-adult transition.

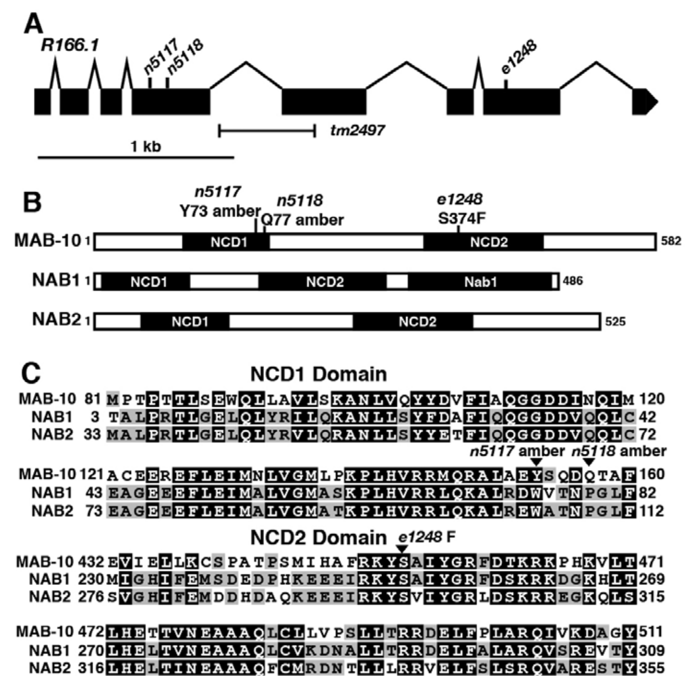

Fig. 2. *mab-10* encodes a NAB transcriptional co-factor. (**A**) The *mab-10* (*R166.1*) open reading frame showing the locations of introns and mutations. (**B**) MAB-10 contains the conserved NCD1 and NCD2 domains that are characteristic of NAB proteins. (**C**) Conservation of the NCD1 and NCD2 domains of MAB-10 and mouse NAB1 and NAB2. Black boxes represent identical regions; gray boxes represent regions of 66% similarity.

MAB-10 and LIN-29 are co-expressed in multiple tissues during development

To determine whether MAB-10 and LIN-29 are co-expressed, we generated rescuing MAB-10::GFP and LIN-29::mCHERRY fusion proteins and performed single-molecule FISH of endogenous *mab-10* transcripts. Expression of LIN-29::mCHERRY largely resembled the previously published pattern of LIN-29 expression (Bettinger et al., 1996). MAB-10 and LIN-29 were co-expressed in multiple cells, including specific pharyngeal cells throughout development (Fig. 3A-C), vulval precursor cells during the third larval stage (Fig. 3D-F), and the seam cells and hypodermal nuclei derived from the seam cell lineages throughout the L4 stage and adulthood (Fig. 3G-I).

lin-29 is required for MAB-10::GFP accumulation in seam cells during the larval-to-adult transition

Because LIN-29 is the master regulator of the larval-to-adult transition, we wanted to determine whether *mab-10* expression was dependent on LIN-29. We expressed MAB-10::GFP in *lin-29(n836)* mutants and found that the pattern of MAB-10::GFP expression was similar to the wild type with the notable exception of the seam cells. During the L4 stage, MAB-10::GFP was present within the nuclei of the syncytial hypoderm but was not visible within the seam cells (Fig. 3J-L).

We also expressed LIN-29::mCHERRY in *mab-10* mutants and found that removal of *mab-10* had no effect on LIN-29::mCHERRY accumulation (Fig. 3M-O). These results demonstrate that *mab-10* probably does not promote *lin-29* activity by regulating *lin-29* expression and suggest that either *mab-10* transcription is controlled by LIN-29 specifically in the seam cells or that LIN-29 promotes MAB-10::GFP nuclear accumulation post-transcriptionally.

mab-10 transcription is not dependent on *lin-29*

To determine whether LIN-29 promotes *mab-10* transcription in the seam cells, we performed single-molecule FISH (Raj et al., 2008) to detect endogenous *mab-10* transcripts in wild-type and *lin-29(n836)* animals.

We observed during the L1 stage *mab-10* transcripts in the anterior and posterior bulbs of the pharynx as well as a small number of transcripts throughout the hypoderm (Fig. 4A). L2-stage animals showed increased expression of *mab-10* mRNA in hyp7 and the rectal epithelium in addition to the nerve ring and the ventral nerve cord, where several cells contained one or two transcripts (Fig. 4B). During the early L3 stage, *mab-10* was weakly expressed in hyp7 but showed high expression in the vulval and uterine precursor cells (Fig. 4C). *mab-10* mRNA was also present within the distal tip cells and a pair of bilaterally symmetric cells that we believe to be the CAN neurons, based on their position and neuronal nuclear morphology. The CAN neurons are associated with the excretory canal and are thought to regulate excretory function (Sulston et al., 1983; Nelson and Riddle, 1984; Forrester et al., 1998).

By the late L3 stage, *mab-10* expression was almost absent from hyp7 but was high in the seam cells, distal tip cells and developing vulva (Fig. 4D). *mab-10* transcript was also detected in the gonadal sheath cells. During the L4 stage, *mab-10* transcripts dramatically increased in abundance throughout the pharynx, hypoderm and somatic gonad, including the distal tip cells (Fig. 4E).

We found no change in the level or pattern of *mab-10* transcription in a *lin-29(n836)* mutant background compared with the wild type (Fig. 4F), suggesting that *mab-10* is not a transcriptional target of LIN-29 and that the absence of MAB-10::GFP from the nuclei of L4 seam cells reflects a post-transcriptional regulation of MAB-10 by LIN-29.

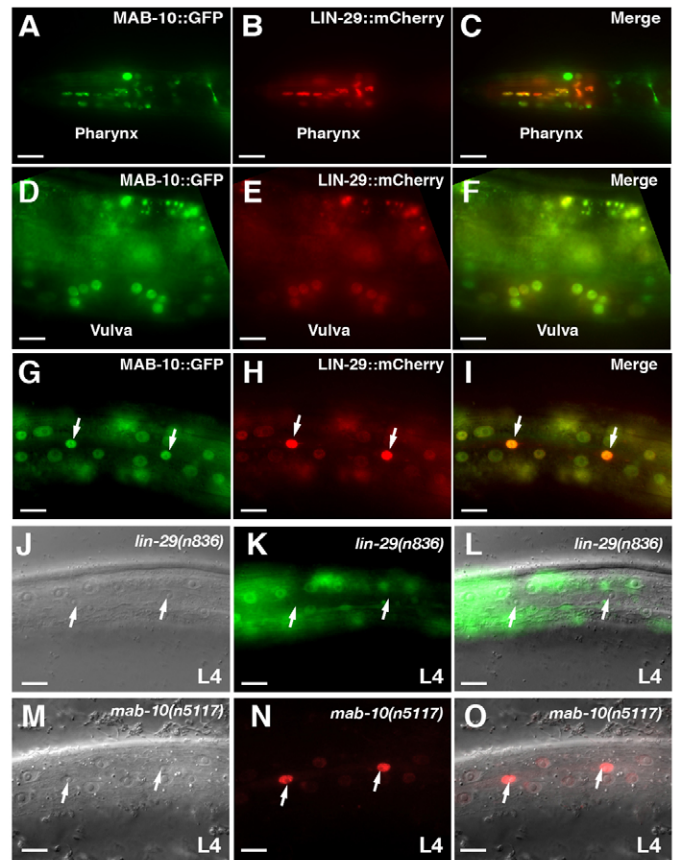

Fig. 3. *mab-10* is co-expressed with *lin-29*; LIN-29 is required for MAB-10 accumulation in the seam cells. (**A-I**) MAB-10::GFP and LIN-29::mCherry proteins localize (A-C) to nuclei of the pharynx throughout development, (D-F) to the nuclei of vulval precursor cells (L4 shown), (G-I) to seam cell nuclei (arrows) and to the majority of the seam-cell-derived hypodermal nuclei during the L4 stage. (**J-L**) Absence of MAB-10::GFP localization in seam cells (arrows) of a *lin-29* mutant. (**M-O**) Unaltered LIN-29B::mCherry expression in an L4 *mab-10(n5117)* hermaphrodite. Arrows indicate seam cell nuclei. Scale bar: 10 μm.

MAB-10 interacts with a region of the LIN-29 C terminus that is conserved in mammalian EGR proteins

Using in vitro pull-down assays, we found that MAB-10::GST interacted with in vitro-translated LIN-29 but did not interact with luciferase (Fig. 5A; see Fig. S3A in the supplementary material). A similar interaction between *Drosophila* NAB and the two *Drosophila* LIN-29 homologs RN and SQZ was reported recently (Terriente Felix et al., 2007). Deletion of a 17 amino acid sequence (390-406) (Fig. 5B; see Fig. S3B in the supplementary material) within the LIN-29 C terminus decreased MAB-10 binding by about 80% (Fig. 5C, lane 3). Alignment of LIN-29 with the closest homologs from the distantly related nematodes *Brugia malayi* and *Pristionchus pacificus* revealed significant identity within only the zinc-finger domains and the region of LIN-29 important for interaction with MAB-10 (see Fig. S4 in the supplementary material). Strikingly, alignment of this 17 amino acid LIN-29

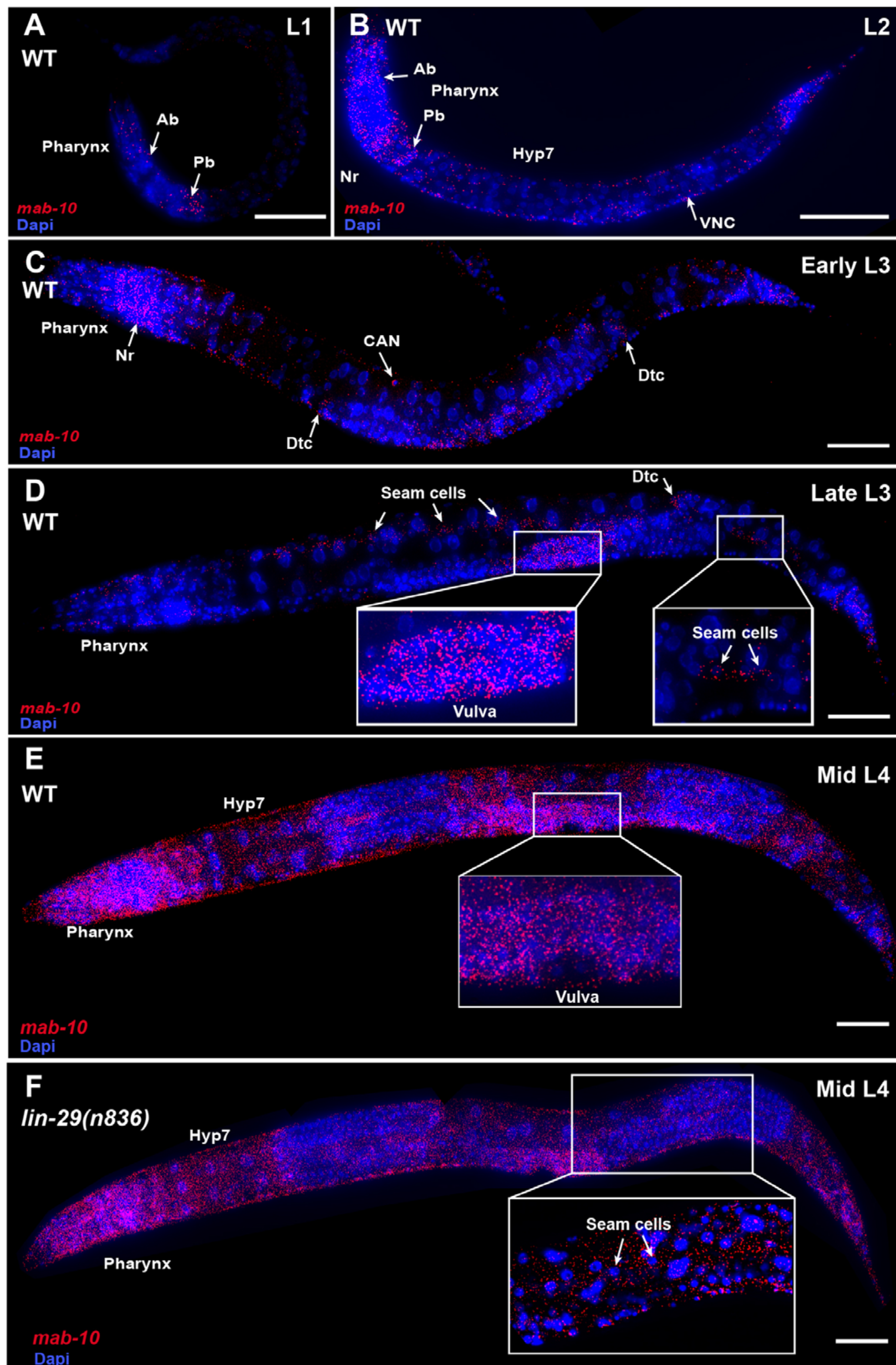

Fig. 4. *mab-10* is expressed in multiple tissues throughout development. Each dot is thought to correspond to one *mab-10* transcript (Raj et al., 2008). Scale bars: 10 μm. (**A**) L1 animal showing expression in the anterior and posterior bulbs (Ab and Pb) of the pharynx and a very low level of expression in the hypoderm. (**B**) L2 animal showing expression in the pharynx, the nerve ring (Nr), the ventral nerve cord (VNC), the rectal epithelium and the hypoderm (hyp7). (**C**) Early L3 animal (Pn.p cells have divided once) showing expression in the pharynx, the nerve ring, the distal tip cells (Dtc), the canal-associated neuron (CAN), and all of the cells of the vulval and uterine precursor cells. There is also a low level of expression throughout hyp7. (**D**) Late L3 animal (Pn.p daughters have divided once) showing reduced expression in the pharynx, and increased expression in the seam cells, developing vulva, uterus and distal tip cells. (**E**) Mid-L4 animal showing high levels of *mab-10* expression throughout the pharynx, hyp7 and gonad. (**F**) Mid-L4 *lin-29(n836)* hermaphrodite showing high levels of *mab-10* expression throughout the hypoderm, including the seam cells.

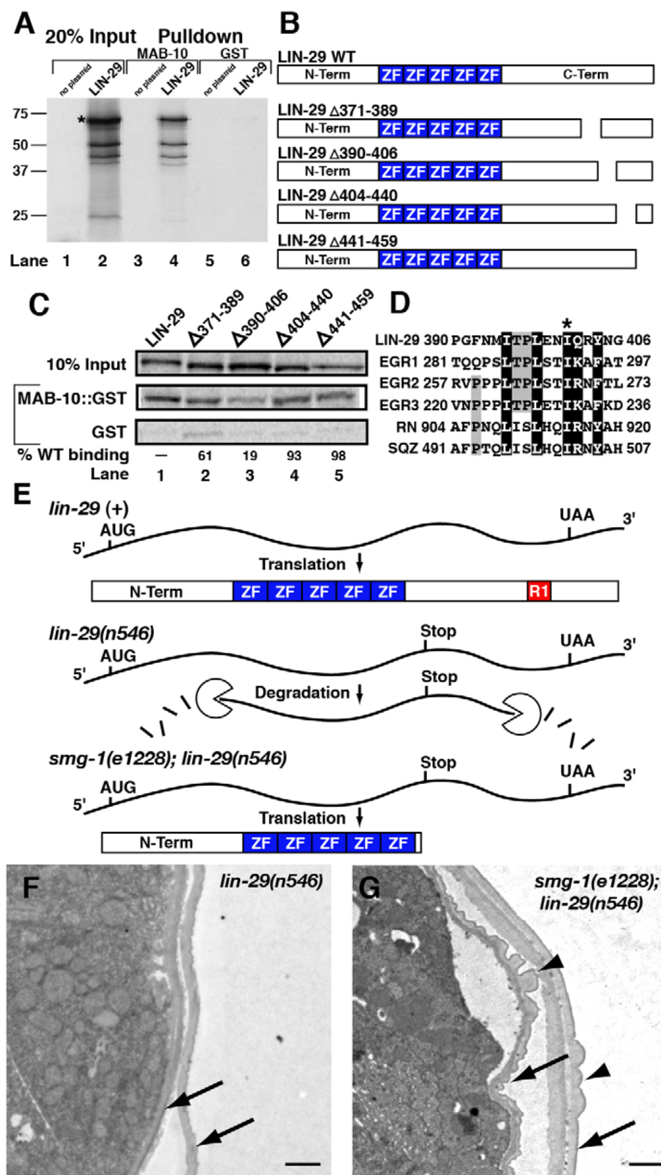

Fig. 5. A conserved domain in the LIN-29 C terminus is required for MAB-10 binding in vitro and for complete terminal differentiation in vivo. (**A**) GST pull-downs using in vitro translated ^{35}S-labeled LIN-29, MAB-10::GST and GST alone. An asterisk indicates full-length LIN-29. (**B**) Diagram of LIN-29 protein and deletion variants. (**C**) GST pull-downs performed with LIN-29 deletion variants and MAB-10::GST. (**D**) Comparison of the MAB-10 interaction domain of LIN-29 with a region of amino acids within the R1 domains of EGR1, EGR2 and EGR3 (*H. sapiens*), and the C termini of RN and SQZ (*Drosophila*) (Terriente Felix et al., 2007). Isoleucine 268 (asterisk) of EGR2 is mutated to asparagine in the recessive form of the familial hypomyelinating neuropathy Charcot-Marie-Tooth disease (Warner et al., 1998). (**E**) The generation of C-terminally truncated LIN-29. *lin-29(n546)* is a C-to-T transition mutation that introduces a premature opal stop codon at amino acid 294, immediately downstream of the zinc-finger-coding region. *lin-29(n546)* mRNA is degraded by nonsense-mediated mRNA decay. Prevention of nonsense-mediated mRNA decay by inactivation of the kinase SMG-1 (Hodgkin et al., 1989) should result in truncated LIN-29 protein. (**F**,**G**) Electron micrographs of cross-sections of adult male *lin-29(n546)* and *smg-1(e1228); lin-29(n546)* animals undergoing an extra molt. Arrows indicate individual cuticles, whereas arrowheads indicate adult-specific lateral alae. Scale bars: 2 μm.

sequence with the R1 domains of mammalian EGR1, EGR2 and EGR3 revealed a conserved motif (Fig. 5D). Furthermore, and unexpectedly, we identified the same conserved region within the domains of *Drosophila* RN and SQZ previously shown to bind to *Drosophila* NAB, but not previously known to be similar to EGR proteins. Mutation of the completely conserved isoleucine (268) to asparagine in this region of EGR2 causes a recessive form of the hypomyelinating neuropathy Charcot-Marie tooth disease in humans (Warner et al., 1998) and eliminates NAB/EGR binding in vitro (Warner et al., 1999). Based on these observations, we conclude that LIN-29, RN, SQZ and the mammalian EGR proteins share a conserved NAB-interacting domain.

The LIN-29 C terminus is required in vivo for complete terminal differentiation during the larval-to-adult transition

To determine whether the LIN-29 C terminus is required in vivo for *lin-29* function, we attempted to generate transgenic animals expressing an engineered form of LIN-29 missing the C terminus or specifically missing the putative MAB-10 interaction domain. These attempts were unsuccessful. As an alternative, we used *lin-29(n546)*, an allele that carries a nonsense mutation immediately following the last zinc-finger domain (Fig. 5E). In an otherwise wild-type background, *lin-29(n546)* animals are completely defective in all four aspects of hypodermal terminal differentiation. However, some *lin-29* activity can be restored to *lin-29(n546)* mutants by disrupting nonsense-mediated mRNA decay using a mutation in a *smg* (suppressor with morphological effect on genitalia) gene (Hodgkin et al., 1989). The lack of nonsense-mediated decay presumably stabilizes the *lin-29(n546)* mRNA and allows for the translation of a LIN-29 product missing the C terminus. An antibody raised against the LIN-29 C terminus was unable to detect LIN-29 protein (A. Rougvie, personal communication), suggesting that the restoration of LIN-29 activity was the result of the production of truncated protein and not the result of the production of full-length LIN-29 protein via read-through of the nonsense mutation.

We characterized the extent of terminal differentiation in *smg-1(e1228)*; *lin-29(n546)* double mutant animals and found them to be normal with respect to seam cell fusion, adult cuticle synthesis and exit from the cell cycle. However, 93% of *smg-1(e1228); lin-29(n546)* males failed to exit the molting cycle and generated an extra adult cuticle (Fig. 5F,G) (Table 1), indicating that the LIN-29 C terminus is required for these events.

RNAi of *lin-29* can cause a *mab-10*-like mutant phenotype

Although the absence of the LIN-29 C terminus causes extra molts, it is not clear whether the extra molts are the result of decreased MAB-10 binding, or of a MAB-10-independent general decrease in *lin-29* function. To address this, we partially decreased *lin-29* function using RNAi. We found that a partial reduction of *lin-29* function could induce an extra molt in males and inappropriate adult seam cell divisions in hermaphrodites, while having little to no effect on adult alae formation (see Fig. S5A-C in the supplementary material). This result demonstrates that a reduction of general *lin-29* activity is sufficient to phenocopy *mab-10* mutants and that adult alae synthesis, seam cell fusion, seam cell exit from the cell cycle and exit from the molting cycle probably require different levels of LIN-29 activity. These results, combined with our observation that *mab-10* promotes precocious adult alae formation in *lin-41* mutants without being required for normal adult

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alae formation, raises the possibility that MAB-10 promotes general LIN-29 activity and does not act specifically on target genes controlling molting cycle exit and seam cell exit from the cell cycle.

mab-10 prevents extra molts by repressing the expression of the nuclear hormone receptors *nhr-23* and *nhr-25* in the adult hypoderm

Previous reports implicated the nuclear hormone receptors NHR-23 (nuclear hormone receptor) and NHR-25/SF1 as the primary positive regulators of molting (Kostrouchova et al., 1998; Asahina et al., 2000; Gissendanner and Sluder, 2000; Frand et al., 2005; Hayes et al., 2006). Both *nhr-23* and *nhr-25* are expressed at high levels in the hypoderm, and it has been proposed that LIN-29 represses their expression during adulthood, preventing the execution of further molts (Hayes et al., 2006). We performed genetic mosaic analysis, the result of which suggested that *mab-10* functions in the hypoderm to prevent extra molts (see Fig. S6 in the supplementary material). We used single molecule FISH to quantify the endogenous expression of *nhr-23* and *nhr-25* in the hypoderm of wild-type, *lin-29* and *mab-10* adults (Raj et al., 2008). As expected, we detected a low number of *nhr-23* and *nhr-25* transcripts in the hypoderm of adult wild-type hermaphrodites and males (Fig. 6A-D). We found no significant increase of *nhr-23* expression relative to the wild type (Fig. 6E,J) in adult *mab-10* hermaphrodites. However, in adult *mab-10* males, *nhr-23* expression was increased 19-fold (Fig. 6F,J). We found a near threefold increase in expression of *nhr-25* in *mab-10* hermaphrodites (Fig. 6G,L) and an 11-fold increase in *mab-10* males (Fig. 6H,L). The difference in *nhr-23* and *nhr-25* expression between *mab-10* hermaphrodites and males correlates with the difference in penetrance of the extra molt defect between the sexes. By comparison, we found that *nhr-23* and *nhr-25* were upregulated about 12-fold relative to the wild type in adult-aged *lin-29* hermaphrodites (Fig. 6I-L). These results are consistent with the hypothesis that MAB-10 acts with LIN-29 to prevent extra molts by repressing the expression of *nhr-23* and *nhr-25* in the adult hypoderm.

MAB-10 prevents extra seam cell divisions by promoting the expression of *cki-1*

During the L4 stage, LIN-29 upregulates the cell cycle inhibitor *cki-1* (cyclin-dependent kinase inhibitor) in seam cells (Hong et al., 1998). *cki-1* and its homolog *p27* promote terminal differentiation in *C. elegans* and mammals, respectively (McArthur et al., 2002; Fukuyama et al., 2003; Thomas et al., 2004; Fujita et al., 2007). We hypothesized that the extra seam cell divisions in *mab-10* mutants might be the result of reduction of *cki-1* expression during the larval-to-adult transition. RNAi of *cki-1* by injection can induce extra larval seam cell divisions (Hong et al., 1998). We found that postembryonic RNAi of *cki-1* by feeding was sufficient to induce inappropriate adult seam cell divisions (Table 1; see Fig. S6D in the supplementary material) without causing extra larval divisions. We used single-molecule FISH to detect endogenous *cki-1* transcripts in wild-type, *mab-10* and *lin-29* animals.

We observed a pulse of *cki-1* expression in all seam cells following normal seam cell divisions in wild-type, *mab-10* and *lin-29* strains. In wild-type animals and *mab-10* mutants, a second pulse of *cki-1* expression occurred late during the L4 stage after the seam cells had fused. This L4-specific increase was not observed in *lin-29* animals, although there was a pulse of *cki-1* expression associated with the extra seam cell divisions. In wild-type and *mab-10* animals, L4 expression was highest in regions of the seam containing those seam cell nuclei least likely to undergo extra divisions in *mab-10* mutants: those at the anterior and posterior ends of the animal, as well as those directly dorsal to the vulva (Fig. 6M,N). The total number of *cki-1* transcripts within the seam of *mab-10* animals (556±70) was on average two-thirds of that found in wild-type animals (863±166). In *lin-29* animals, the seam cells underwent an extra cell division and displayed the typical pattern of seam cell *cki-1* expression observed following a normal cell division. These observations are consistent with the hypothesis that MAB-10 acts with LIN-29 to prevent extra seam cell divisions by promoting the expression of *cki-1*.

DISCUSSION

MAB-10 acts with LIN-29 to promote differentiation

We have identified *mab-10* as a new heterochronic gene that is required for specific aspects of the larval-to-adult transition, specifically molting cycle exit and seam cell exit from the cell cycle. We cloned *mab-10* and found that it encodes the only *C. elegans* NAB transcriptional co-factor. NAB proteins are thought to physically interact with Kruppel family EGR transcription factors to regulate their activity.

Previous work demonstrated that MAB-10 (then known only as the *C. elegans* NAB protein R166.1) could interact with mammalian EGR proteins in a yeast two-hybrid assay; no corresponding *C. elegans* EGR protein was identified (Svaren et al., 1996). We demonstrate that MAB-10 interacts with the terminal differentiation factor LIN-29 through an evolutionarily conserved NAB binding domain (R1 domain) and that MAB-10 is required for a subset of LIN-29-dependent activities. Our work identifies LIN-29 as a *C. elegans* EGR-like protein and demonstrates that the *C. elegans* heterochronic pathway controls the timing of NAB/EGR-mediated differentiation.

Several experiments using mammalian tissue culture suggest that NAB proteins negatively regulate EGR activity by binding EGR proteins at specific target genes and preventing EGR-mediated transcription (Russo et al., 1995; Svaren et al., 1996). However, loss of either EGR2 function or NAB function in mice and humans results in hypomyelination, suggesting that EGR and NAB proteins need not act antagonistically in vivo.

We found that in *C. elegans*, MAB-10 and LIN-29 both act to promote terminal differentiation and the onset of adulthood. Furthermore, we showed that *mab-10* promotes the formation of precocious adult alae in a *lin-41* mutant background, suggesting that MAB-10 does not specifically act to control genes required for exit from the molting cycle and seam cell exit from the cell cycle, but more likely acts as a general enhancer of LIN-29 activity.

LIN-29 probably promotes MAB-10 seam cell nuclear accumulation post-transcriptionally

EGR and NAB proteins have been shown to operate in a negative-feedback loop wherein an EGR protein promotes the expression of its NAB co-factor, which then inhibits EGR activity (Kumbrink et al., 2005). We found that *mab-10* transcription does not depend on LIN-29, despite a dramatic increase of *mab-10* transcription during the L4 stage. Thus, *mab-10* is not a transcriptional target of LIN-29.

Whereas *mab-10* is not a transcriptional target of LIN-29, MAB-10::GFP localization to seam cell nuclei during the L4 stage required LIN-29, indicating that LIN-29 might promote MAB-10 seam cell nuclear localization via a post-transcriptional mechanism or via direct physical interaction.

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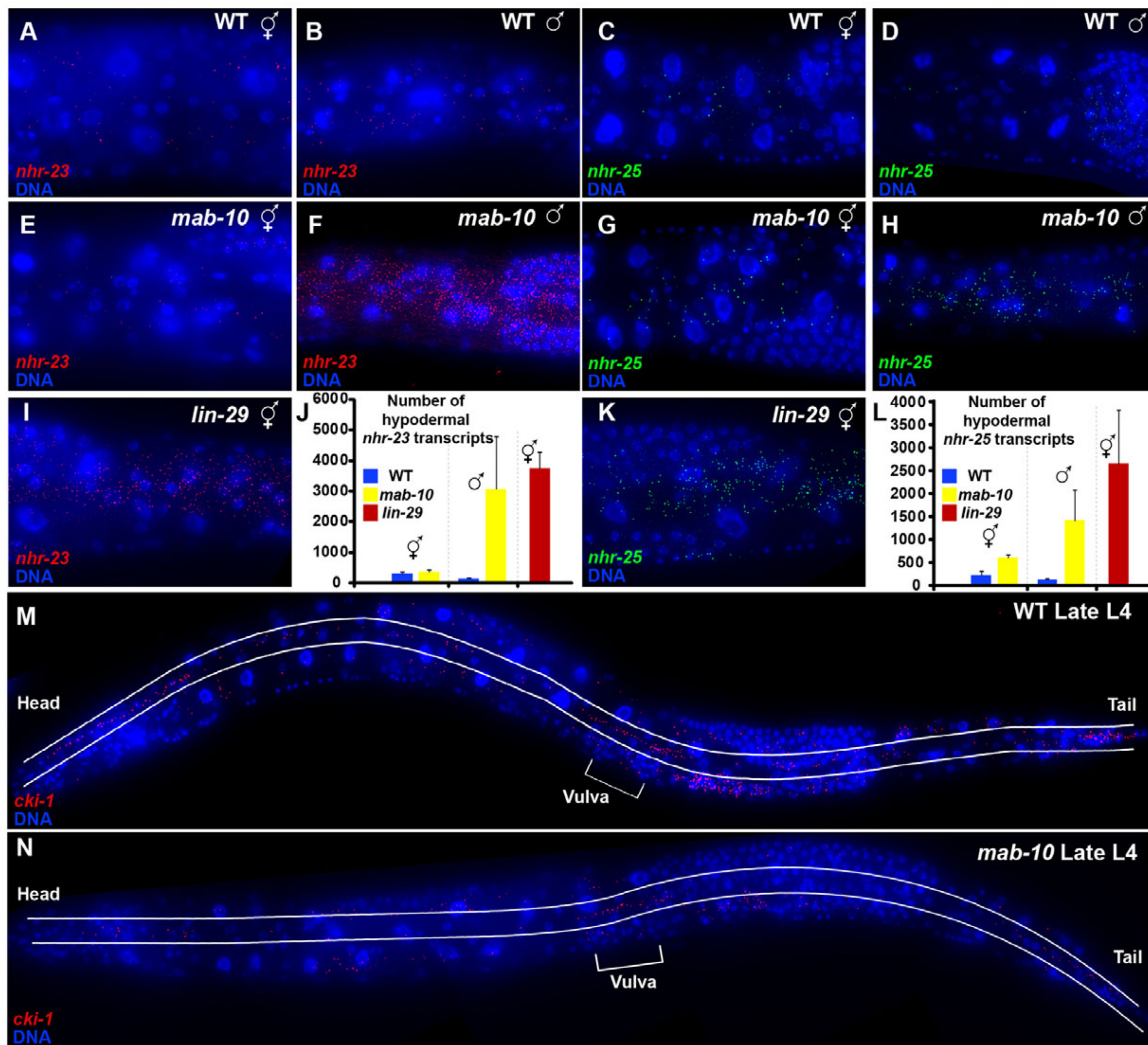

Fig. 6. MAB-10 and LIN-29 control the expression of *nhr-23*, *nhr-25* and *cki-1* during the larval-to-adult transition. (**A-D**) Micrographs showing hypodermal expression of *nhr-23* and *nhr-25* in wild-type (WT) adult hermaphrodites and males, respectively. (**E-H**) Micrographs showing hypodermal expression of *nhr-23* and *nhr-25* in *mab-10(n5117)* adult hermaphrodites and males, respectively. (**I**) Hypodermal expression of *nhr-23* in adult-aged *lin-29* hermaphrodite. (**J**) Quantification of hypodermal *nhr-23* expression in wild-type, *mab-10* and *lin-29* adults [mean±s.d., wild-type hermaphrodites, 303±57 (*n*=7); wild-type males, 161±13 (*n*=5); *mab-10* hermaphrodites, 360±71 (*n*=8); *mab-10* males, 3082±1718 (*n*=7); *lin-29* hermaphrodites, 3748±533 (*n*=7)]. (**K**) Hypodermal expression of *nhr-25* in adult-aged *lin-29* hermaphrodite. (**L**) Quantification of hypodermal *nhr-25* in wild-type, *mab-10* and *lin-29* adults [mean±s.d., wild-type hermaphrodites, 226±92 (*n*=18); wild-type males 125±29 (*n*=8); *mab-10* hermaphrodites, 613±67 (*n*=8); *mab-10* males, 1442±661 (*n*=11); *lin-29* hermaphrodites, 2653±1177 (*n*=8)]. (**M**,**N**) Seam cell expression of *cki-1* in late L4 wild-type (868±180, *n*=9) and *mab-10* (556±70, *n*=10) hermaphrodites ($P<0.0005$ by one-tailed *t*-test for samples with unequal variance). Two parallel white lines indicate the lateral seam. Note the abundance of *cki-1* transcript at the anterior and posterior ends of the animal, as well as directly dorsal to the vulva.

Our work demonstrates that MAB-10 and LIN-29 do not operate in a negative-feedback loop. We propose that other components of the heterochronic pathway directly regulate *mab-10* transcription to temporally regulate MAB-10/LIN-29 activity and that LIN-29 or some factor downstream of LIN-29 controls MAB-10/LIN-29 activity by promoting the accumulation of MAB-10 in seam cell nuclei.

Homologs of MAB-10, LIN-29, LIN-28 and NHR-25 regulate cellular differentiation and the onset of adulthood in mammals

By showing that MAB-10 acts with LIN-29 through an evolutionarily conserved EGR R1 domain, we identify LIN-29 and the *Drosophila* LIN-29 homologs RN and SQZ as EGR-like molecules. We propose that NAB proteins and EGR proteins act together in temporal developmental programs to control terminal differentiation. In *Drosophila*, the LIN-29 homolog SQZ acts with *Drosophila* NAB to control neuroblast differentiation (Baumgardt et al., 2009). We showed that in *C. elegans*, LIN-29 and MAB-10 act together to control the differentiation of a hypodermal stem cell lineage during the transition from larva to adult by regulating the expression of the nuclear hormone receptors *nhr-23* and *nhr-25* and the cell cycle regulator *cki-1* (Fig. 7A). Recently, a study of *C. elegans* demonstrated that *nhr-25* is itself a heterochronic gene and possibly functions with *lin-29* to promote aspects of the larval-to-adult transition, including seam cell exit from the cell cycle (Hada et al., 2010). Though the mechanism by which *nhr-25* regulates

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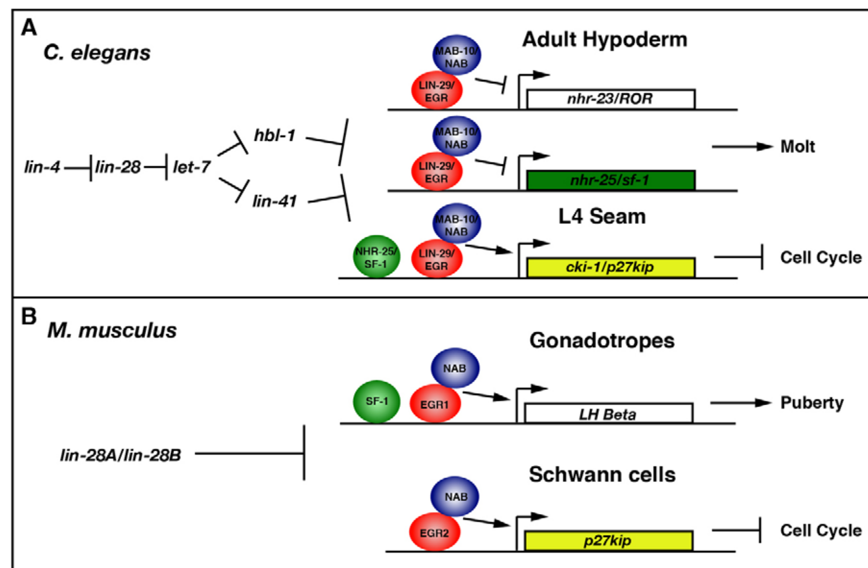

Fig. 7. Pathways that control the onset of adulthood and the terminal differentiation of specific cell lineages are conserved from *C. elegans* to mammals. (**A**) The *C. elegans* heterochronic pathway controls hypodermal terminal differentiation and the onset of adulthood by regulating the timing of MAB-10/LIN-29 activity. In the adult hypoderm, MAB-10 and LIN-29 prevent the expression of the nuclear hormone receptors *nhr-23* and *nhr-25*, which promote the expression of downstream genes required for molting. In the L4 seam cells, MAB-10 and LIN-29 prevent extra seam cell divisions by promoting the expression of the cell-cycle inhibitor *cki-1/p27kip*. NHR-25 has been proposed to act with LIN-29 to promote seam cell exit from the cell cycle. (**B**) Above, a model proposing that mammalian LIN28 controls the onset of menarche by regulating the timing of NAB/EGR activity in the gonadotrope lineage, possibly via a conserved heterochronic pathway. The nuclear hormone receptor SF1/NHR25 acts with EGR1 to promote the expression of the luteinizing hormone β subunit. Below, a model proposing that mammalian LIN28 controls the terminal differentiation of Schwann cells by regulating NAB/EGR activity.

seam cell exit from the cell cycle is not known, we speculate that LIN-29 and NHR-25 might act together to promote *cki-1* expression (Fig. 7A).

EGR proteins were originally identified as immediate-early genes and generally have been regarded as differentiation factors. Like *mab-10* and *lin-29* mutants, Nab and Egr mutant mice are defective in the terminal differentiation of several cell lineages. For example, in Schwann cells, EGR2 promotes the expression of P27, the homolog of *C. elegans* CKI-1, and acts with NAB proteins to promote terminal differentiation (Fig. 7B) (Parkinson et al., 2004; Baloh et al., 2009). Mammalian homologs of other *C. elegans* heterochronic genes also control differentiation. Similar to the role of LIN-28 in *C. elegans*, mammalian LIN28 and LIN28B promote stem cell identity and prevent differentiation by repressing the *let-7* microRNA gene (Yu et al., 2007; Heo et al., 2008; Viswanathan et al., 2008). As in *C. elegans*, increasing levels of *let-7* drive differentiation, and the mouse homolog of LIN-41, LIN41, has been shown to be a *let-7* target acting in stem cell niches to prevent premature differentiation (Rybak et al., 2009).

Mammalian LIN-28 controls the timing of the onset of puberty in mice and possibly humans (Fig. 7B) (Ong et al., 2009; Zhu et al., 2010). Mice lacking EGR1 function, like *lin-29* mutants of *C. elegans*, fail to undergo puberty (Topilko et al., 1998). EGR1 and NAB proteins act with SF1, the homolog of *C. elegans* NHR-25, in the gonadotrope lineage of the pituitary gland to regulate the expression of luteinizing hormone and the onset of puberty (Topilko et al., 1998). The molecular mechanism by which mammalian LIN-28 regulates the onset of puberty is not known. Our work raises the possibility that homologs of *C. elegans* heterochronic genes might act in an evolutionarily conserved pathway that controls the terminal differentiation of cell lineages and the onset of adulthood by regulating the activity of NAB and EGR proteins (Fig. 7B).

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Competing interests statement
The authors declare no competing financial interests.

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