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The *C. elegans* Mi-2 chromatin-remodelling proteins function in vulval cell fate determination

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SUMMARY

The Mi-2 protein is the central component of the recently isolated NuRD nucleosome remodelling and histone deacetylase complex. Although the NuRD complex has been the subject of extensive biochemical analyses, little is known about its biological function. Here we show that the two *C. elegans* Mi-2 homologues, LET-418 and CHD-3, play essential roles during development. The two proteins possess both shared and unique functions during vulval cell fate determination, including antagonism of the Ras signalling pathway required for vulval cell fate induction and the proper execution of the 2° cell fate of vulval precursor cells, a process under the control of LIN-12 Notch signalling.

Key words: *Caenorhabditis elegans*, Mi-2, Development, Vulva, Ras, Notch, NuRD

INTRODUCTION

The Mi-2 protein, originally identified as an autoantigen of the human disease dermatomyositis (Seelig et al., 1995; Woodage et al., 1997), belongs to the highly conserved CHD family of proteins which, in addition to an ATPase/helicase domain of the SWI2/SNF2 class, also contains two PHD zinc-finger motifs, two chromo domains, and a truncated helix-turn-helix DNA-binding motif with limited similarity to the telobox DNA-binding domain (Woodage et al., 1997). Homologues of the Mi-2 protein have been identified in vertebrates, *Drosophila*, yeast and plants (Delmas et al., 1993; Woodage et al., 1997; Kehle et al., 1998; Eshed et al., 1999).

Recently, the Mi-2 protein has been shown to be a component of the nucleosome remodelling and histone deacetylase complex NuRD isolated from human cell lines and *Xenopus* egg extracts (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998; Wade et al., 1998; Wade et al., 1999). Extensive biochemical analysis of this complex has shown that, in addition to Mi-2, it contains the two histone deacetylases HDAC1 and HDAC2, the histone-binding proteins RbAp48/46, an MTA1-related protein, and the methyl binding protein MBD3. It has been hypothesized that the biochemical association of histone deacetylase and nucleosome remodelling activities is required for efficient histone deacetylation in vivo and facilitates dynamic changes in nucleosomal structure. Thus, the NuRD complex may establish or maintain hypoacetylated chromatin domains, thereby allowing transcriptional repression of target genes (Xue et al., 1998; Zhang et al., 1999). The biological functions and the targets of the Mi-2 proteins and the Mi-2 containing NuRD complex are just beginning to be characterized (for a recent review see Ahringer, 2000).

To gain further insight into the biological function of the Mi-2 proteins during development, we have undertaken a genetic analysis of the two *C. elegans* Mi-2 homologues. We found that *let-418* and *chd-3* have essential and partially redundant functions during development. In particular, we have characterized two specific roles played by these genes in cell fate determination during vulval development. We show that one of the *C. elegans* Mi-2 homologues, LET-418, plays a role in antagonizing the RTK/Ras/MAP kinase pathway via the synthetic multivulva (synMuv) pathway, supporting the recently proposed link between chromatin remodelling by NuRD-like complexes and the Ras signalling pathway (Solari and Ahringer, 2000; Walhout et al., 2000). Furthermore, we find that LET-418 and CHD-3 appear to have a shared role in the proper execution of the LIN-12 Notch dependent 2° cell fate of the P5.p and P7.p vulval precursor cells.

MATERIALS AND METHODS

General methods

The cDNA sequences of *chd-3* and *let-418* were obtained starting from the cDNAs yk172f1 (*let-418*) and yk47h5 (*chd-3*). The 5′ and 3′ ends of each cDNA were amplified by PCR from the λRB2 cDNA

library and a poly(T)-primed cDNA library, pAE1, respectively. The molecular lesions associated with the different *let-418* and *chd-3* mutations were determined by sequencing of both strands of the genomic regions amplified from a pool of homozygous animals. Each mutation was confirmed by sequencing two independent PCR reactions.

In situ RNA hybridization

In situ RNA hybridization experiments were performed according to Seydoux and Fire (1995). Primers used for PCR on genomic DNA were 5′-GAGGAGTGCAAGCAAGATGG-3′ and 5′-CGGATGTCA-ATCTTCTCACG-3′ for *let-418* and 5′-CAAGATGGCGAGCTTA-TGC-3′ and 5′-TCGAGGTGACTTCATCCTCC-3′ for *chd-3*. The PCR products were subcloned in the pGEM-T vector (Promega) to generate single-stranded probes. The post-hybridization washes were as described, but for 30 minutes at 60°C, 3× 30 minutes at 48°C and 20 minutes at room temperature.

RNA interference

RNAi experiments were carried out as described by Fire et al. (1998). Antisense and sense RNA were produced using an in vitro transcription kit (Promega). For *let-418* and *chd-3* the same PCR-derived clones used for in situ hybridization experiments (see above) were used as templates for in vitro transcription. For *lin-15A*- and *lin-15B*-specific RNAi we used the following primers for PCR on genomic DNA: 5′-CATCTCGGAGACGGAAAATC-3′ and 5′-GTT-GGCGGAATATGTTTTGG-3′ (*lin-15A*); 5′-ACCTGAGCCAGAG-AGAAACG-3′ and 5′-GGCACGTATTCGCAATCTTC-3′ (*lin-15B*). The PCR products were subcloned into the pGEM-T vector (Promega) and used for in vitro transcription as above. After each experiment, the efficiency of RNAi was confirmed by using the same needle to inject animals mutant for a synMuv gene from the complementary class and scoring F_1 progeny for the presence of a Muv phenotype.

Strains and genetics

Worms were grown on NGM plates at 20°C as described (Brenner, 1974). Mutations used were as follows. LGII, *lin-8(n111), dpy-10(e128), lin-38(n751), unc-52(e444), let-23(sy97) unc-4(e120)*; LG III, *lin-9(n112) dpy-17(e164), lin-36(n766) dpy-19(e1259)*; LGIV, *lin-3(n378)*; LGV, *unc-46(e177), let-418(s1617), let-418(ar113), let-418(ar114), dpy-11(e224), sDf44*; LGX, *lin-15A(n767), lin-15B(n374), chd-3(eh4)* (four times backcrossed). For *chd-3;lin-8* and *chd-3;lin-9* double mutants, which did not result in a synMuv phenotype, the presence of the silent synMuv mutation was confirmed by backcrossing to lin-9/+ or lin-8/+ males, respectively, and checking for the presence of Muv animals in the F_2 progeny. Pn.p cell lineages were determined by direct observation of the cell divisions using Nomarski optics, as described previously (Sternberg and Horvitz, 1986).

GFP transgenes

To generate the *let-418*::GFP reporter, the genomic *Sal*I-*Bam*HI fragment containing 11184 bp of promoter region and 181 bp of coding sequence was subcloned into the *Sal*I-*Bam*HI site of pPD95.69 (A. Fire, http://www.ciwemb.edu/pages/firelab.html). This construct was injected into *unc-119(e2498)* animals at 60 ng/μl together with 20 ng/μl of wild-type *unc-119* plasmid (Maduro and Pilgrim, 1995).

RESULTS

The genome of *C. elegans* encodes two Mi-2 homologues

A BLAST search of the *C. elegans* genome identified two open reading frames, T14G8.1 and F26F12.7 (previously named *chd-3* and *chd-4*, respectively, Solari and Ahringer, 2000; but see below), which encode polypeptides highly similar to the human Mi-2 proteins. The corresponding cDNAs appear to be complete as they contain 5′-SL1 splice leader sequences and poly(A) tails at their 3′ ends. The structure of the two genes was assembled by comparing cDNA and genomic sequences (Fig. 1). The two predicted proteins have a length of 1829 (F26F12.7) and 1787 amino acids (T14G8.1), respectively, and contain all of the highly conserved functional domains of the CHD protein family (Fig. 1). They are 71% identical to one another and approximately 59% identical to the human Mi-2 proteins throughout their entire length.

The two *C. elegans* Mi-2 homologues have essential and partially redundant developmental functions

To gain insight into the function of F26F12.7, we first used RNA-mediated interference (RNAi), which has been shown to produce a specific phenocopy of the loss-of-function phenotype of the targeted gene (Fire et al., 1998; Tabara et al., 1998). Injection of dsRNA corresponding to the open reading frame F26F12.7 resulted in a developmental arrest at the first larval (L1) stage (data not shown), suggesting that F26F12.7 encodes an essential gene. This observation prompted us to look for previously defined lethal mutations in the appropriate genetic region. F26F12.7 maps to the center of chromosome V, within a region that includes several candidate lethal mutations. We found that H03G13, a cosmid that contains the full-length sequence of F26F12.7, was able to partially rescue the highly penetrant sterility and the maternal effect L1 arrest of animals homozygous for *evl-11(ar114)* (Seydoux et al., 1993). In addition, another candidate mutation, *let-418(s1617)* (Johnson and Baillie, 1991), failed to complement *evl-11(ar114)*, indicating that the two mutations are allelic. Sequencing of three alleles of *evl-11* and *let-418* showed that they all introduce stop codons in the F26F12.7 gene (Fig. 1B). Based on these data, we conclude that F26F12.7, previously named *chd-4* by Solari and Ahringer (2000), is identical to *let-418* and *evl-11*, and the gene will now be called *let-418*.

Whole-mount in situ hybridization experiments showed that *let-418* mRNA is maternally delivered to the early embryo. *let-418* mRNA was detected in the cytoplasm in most if not all cells from the one-cell stage embryo on (Fig. 2A-C). Control experiments with a *let-418* sense probe detected no signal (Fig. 2D). To gain insight into the function of *let-418*, we analyzed the phenotype of *let-418* mutant animals. Hermaphrodites homozygous for the alleles *ar113*, *ar114* or *s1617*, and inheriting a maternal dose of wild-type activity, showed identical phenotypes, developing into sterile adults. In addition, about 30% of these sterile animals had an everted vulva (Evl) phenotype (Table 1 and Fig. 3A). All vulva precursor cells (VPCs) had a wild-type lineage (Table 2C), with the exception of approximately 7% of homozygous animals from all three alleles which developed a pseudovulva posterior to the vulva (Fig. 3A), the result of an induced fate of the P8.p cell (Table 2A,B). About 2% of the homozygous animals segregating from *let-418*/+ mothers escaped sterility and developed into fertile animals (Table 1). They produced 3-5 progeny without maternal *let-418* contribution, which all arrested at L1 (Table 1). In summary, the phenotype of *let-418* animals is L1 arrest (when both maternal and zygotic activities are removed) or sterile Evl (when only the zygotic activity is missing).

Table 1. Phenotypes of *let-418* and *chd-3* animals

Genotype	Genotype of mother	Phenotype
let-418//*let-418*	*let-418*/+	98% sterile, 2% fertile; everted vulva (30%); 7% induced P8.p
let-418/*let-418*	*let-418*/*let-418*	L1 arrest
chd-3/*chd-3*	*chd-3*/*chd-3*	No obvious phenotype
let-418/*let-418*; *chd-3*/*chd-3*	*let-418*/ +; *chd-3*/*chd-3*	L4 arrest; vulval defects (see text)
chd-3/*chd-3*; *let-418* (RNAi);	*chd-3*/*chd-3*	Embryonic arrest

The three *let-418* alleles *ar114*, *ar113* and *s1617* contain premature stop codons that are predicted to result in severely truncated LET-418 proteins (Fig. 1A). Furthermore, the Evl phenotype and the sterility of animals carrying the allele *ar114* in trans to *sDf44*, a small deficiency that uncovers *let-418*, were identical to that of animals homozygous for *ar114*, *ar113* or *s1617*. Thus, by both genetic and molecular criteria, *ar113*, *ar114* and *s1617* are likely to be strong loss-of-function, if not null, alleles. In the following experiments, *let-418* mutants are *let-418(ar114)* homozygous animals derived from heterozygous mothers.

In contrast to *let-418*, *chd-3* (T14G8.1) mRNA appears not to be maternally delivered, as shown by in situ hybridization on early embryos (Fig. 2E). *chd-3* mRNA was first weakly detected in about 16-cell stage embryos and appeared strongly in 28-cell embryos (Fig. 2F). To investigate the function of *chd-3*, we analyzed a deletion allele, *eh4* (received from the Sanger Center Gene Knockout Consortium), which removes 2018 bp of the *chd-3* genomic sequence (Fig. 1B). The deletion causes a frame shift, resulting in a stop codon after the addition of 12 ectopic amino acids. The predicted mutant protein product lacks almost the entire helicase and putative DNA binding domains (Fig. 1B). By analogy to the three alleles of *let-418*, *chd*-3(*eh4*) is also likely to be a strong loss-of-function or null allele. However, no obvious defects were detected in *chd-3* homozygous mutants (Table 1). A requirement for *chd-3* function became apparent in *chd-3;let-418* double mutants. While homozygous *let-418* animals derived from *let-418*/+ mothers develop into sterile adults (see above), *chd-3;let-418* double mutants with a maternal *let-418* contribution arrested as L3 or L4 larvae (Table 1). Furthermore, *let-418*;*chd-3* double mutants in which maternal *let-418* contribution was depleted by RNAi, arrested as embryos (Table 1). Altogether, these genetic data support our molecularly based assumption that *chd-3*(*eh4*) is a loss-of-function or null allele and suggest that *let-418* and *chd-3* have essential and partially redundant functions during development.

Expression patterns of LET-418 and CHD-3

The expression patterns of *chd-3* and *let-418* were analyzed using green fluorescent protein (GFP) reporter constructs under the control of their endogenous promoters. Both reporter genes had a very similar expression pattern and were expressed in most if not all cells of the embryo (Fig. 4A and data not shown). During larval development and in adults, expression of both reporters was observed in the nuclei of many cells, including the ventral nerve cord cells and the VPCs, the surrounding hypodermal cells and cells of the head and tail regions (Fig. 4C and data not shown).

let-418, but not *chd-3*, is a synMuvB gene

The fact that both *C. elegans* Mi-2 genes are coexpressed in the VPCs raised the possibility that *chd-3* and *let-418* might be required for vulval development. During vulval development each of the six equivalent VPCs, P3.p-P8.p adopt one of three different cell fates (reviewed in Greenwald, 1997; Kornfeld, 1997). The so-called primary (1°), secondary (2°) and tertiary (3°) cell fates differ by their pattern of cell division and by the morphology of their descendants. The adult vulva is formed from the 22 descendants of P5.p, P6.p and P7.p. The centrally located P6.p cell expresses a 1° cell fate that is characterized by three rounds of division giving rise to eight vulval cells, which do not adhere to the ventral cuticle. The flanking P5.p and P7.p adopt a 2° cell fate generating four adhering and three nonadhering descendants. In wild-type animals P3.p, P4.p and P8.p each adopt a 3° cell fate giving rise to two nonvulval cells that fuse to the hyp7 hypodermal syncytium.

The fates of the VPCs are specified by extracellular signals. A LIN-3 EGF-like inductive signal, produced by the anchor cell in the somatic gonad and transduced by the LET-23 RTK/Ras/MAP kinase signalling cascade, causes P5.p-P7.p to adopt a vulval (1° or 2°) fate (reviewed in Kornfeld, 1997; Sternberg and Han, 1998). This signal overcomes inhibitory signals from two functionally redundant sets of genes, known as synMuvs, that antagonize RTK/Ras/MAP kinase signalling in the VPCs (reviewed in Fay and Han, 2000). The synMuv genes fall into two classes, referred to as A and B, that define two functionally redundant pathways. Animals carrying both a class A and a class B mutation exhibit a multivulva (Muv) phenotype because P3.p. P4.p and P8.p adopt induced (1° or 2°) vulval fates, while animals carrying one or more mutations of the same class have a wild-type vulva (Ferguson and Horvitz, 1989). A third signal, referred to as lateral signal, is produced by P6.p expressing the 1º fate and causes the flanking cells, P5.p and P7.p, to adopt the 2º fate via the LIN-12 Notch receptor (Greenwald et al., 1983; Koga and Ohshima, 1995; Simske and Kim, 1995; Ambros, 1999).

The occasional induction of P8.p seen in 7% of the homozygous *let-418* animals (Tables 1 and 2, Fig. 3A) prompted us to test whether *let-418* and *chd-3* might be acting in one of the two synMuv pathways. This possibility was also indicated by the fact that the synMuvB pathway includes two genes encoding LIN-53 RbAp48 and HDA-1, two homologues of vertebrate NuRD subunits (Lu and Horwitz, 1998). We found that animals carrying a mutation in *let-418(ar114)* and a class A gene, *lin-15(n767)* or *lin-38(n751)*, displayed a highly penetrant synMuv phenotype, with high frequencies of ectopic induction of P3.p, P4.p and P8.p (Table 2A,B; Fig. 3B). The vulval cell lineages of four *let-418*(*ar114*); *lin-15A*(*n767*) mutant animals are shown in Table 2C. An identical result was observed in *let-418*(*ar113*); *lin-15A*(*n767*) double mutant animals (data not shown), indicating that the synMuvB phenotype is due to the presence of mutations in *let-418* as opposed to background mutations

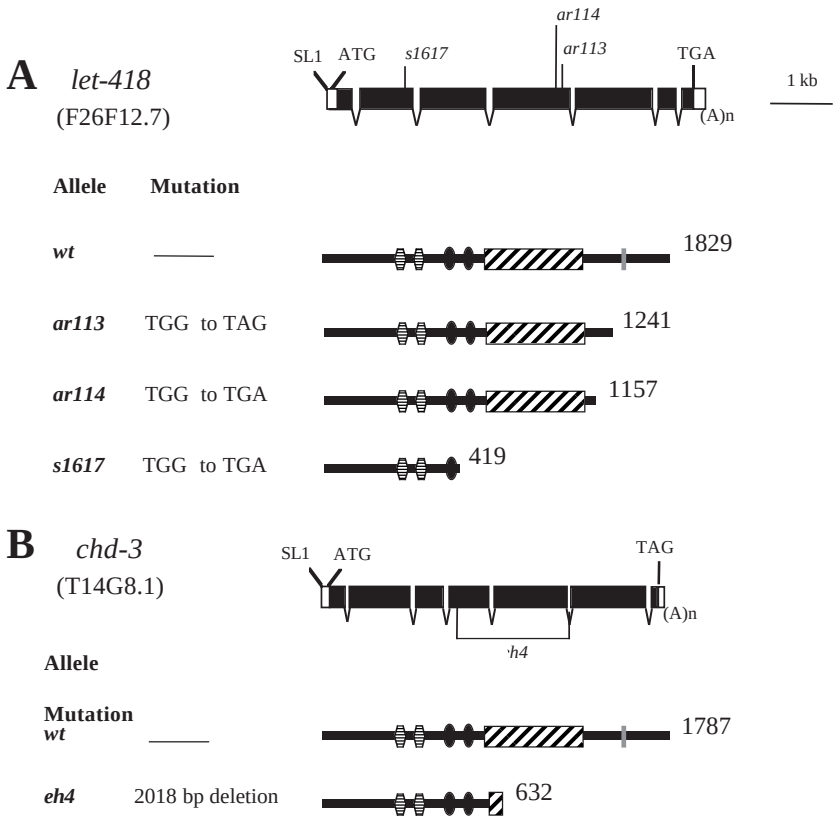

Fig. 1. Gene structure of *let-418*, previously known as *chd-4* (Solari and Ahringer, 2000) (A) and *chd-3* (B). At the top the cDNA structure is shown for each gene. Solid boxes indicate coding sequences, open boxes non-coding sequences, and connecting lines introns. The *trans*-spliced leader SL1, the initiation and stop codons, and the poly(A) tail are shown. Mutational changes for the sequenced alleles are also indicated. Below are schematic diagrams of the CHD-3 (B) and LET-418 (A) proteins with conserved domains represented as filled shapes: PHD zinc fingers, horizontal stripes; chromo domains, solid black; helicase domain, diagonals; telobox related motif, stippled gray. Below the wild-type protein are shown the predicted mutant proteins for each allele with its length in amino acids. The sequence data are available from GenBank under the accession numbers AF308444 (*chd-3*) and AF 308445 (*let-418*).

Table 2. Synthetic vulval phenotypes of *let-418* and *chd-3*

A	Genotype	synMuv*	P8.p induction alone	*n*
let-418	*let-418(ar114)*	0%	7%	56
let-418;class A	*let-418 (ar114); lin-15A(n767)*	82%	n.d.	89
	let-418 (ar114);lin-38(n751)	73%	n.d.	52
	let-418(ar114);lin-15A(RNAi)	80%	n.d.	150
let-418;class B	*let-418 (ar114); lin-15B(n374)*	0%	20%	90
	let-418(ar114); lin-36(n766)	0%	13%	62
	let-418(ar114); lin-15B(RNAi)	0%	n.d.	100
Epistasis	*let-418(ar114); lin-15A(n767); let-23(sy97)*	0%	0%	10

B	% induction of individual VPCs						
Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	*n*
Wild type	0	0	100	100	100	0	Many
lin-15A(n767)	0	0	100	100	100	0	Many
let-418(ar114)	0	0	100	100	100	7	43
let-418(ar114);lin-15(n767)	67	70	100	100	100	71	46
chd-3(eh4)	0	0	100	100	100	0	20
let-418(ar114);chd-3(eh4)	0	4	100	100	100	25	56

C							
Wild type	SS	SS	LLTN	TTTT	NTLL	SS	
let-418(ar114);lin-15A(n767)	LLLN	OOOO	LLLN	TTTT	NOLL	LOOO	1
let-418(ar114);lin-15A(n767)	OTTT	LLOO	LLTN	TTTT	NTLL	OTTT	1
let-418(ar114);lin-15A(n767)	LLTN	SSOO	LLTN	TTTT	NLLL	OOOO	1
let-418(ar114);lin-15A(n767)	S	SS	LLTN	TTTT	NTLL	SS	1
let-418(ar114)	SS	SS	LLTN	TTTT	NTLL	SS	2

(A) *let-418* is a B class synMuv gene.

*The ectopic induction of P8.p alone was not scored as a synthetic multivulva (synMuv) phenotype because this is an inherent feature of *let-418* single mutants (see text).

n, number of animals scored; n.d., not determined.

(B) % induction of individual VPCs was determined by scoring detachment from the cuticle of the Pn.p descendants at the L4 stage.

(C) Cell lineage analyses of *let-418* and *let-418; lin-15(n767)* animals. Pn.p cell lineages are indicated following the nomenclature of Sternberg and Horvitz (1989); T, the nucleus divides transversally (left-right); L, the nucleus divides longitudinally (anterior-posterior); N, the nucleus does not divide; O, nucleus divides obliquely; S, cell fuses with the hypodermal syncytium. Induced (1° or 2°) Pn.p cell fates are boxed.

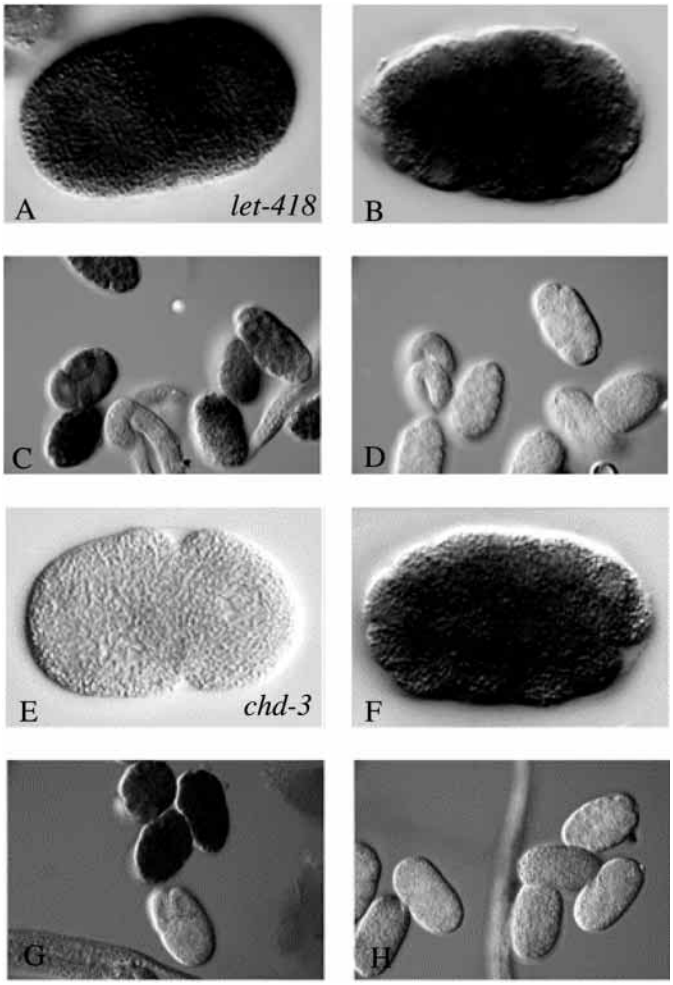

Fig. 2. Localization of *let-418* and *chd-3* mRNAs. Embryos were examined by in situ hybridization. Positive staining is indicated by the dark color. (A-C) embryos were hybridized with a *let-418* antisense probe. (A) *let-418* maternal RNA is detected in 2-cell embryos. (B) In 28-cell embryos, hybridization appears to be uniform in all cells. (E-G) embryos were hybridized with antisense probe to *chd-3*. (E) No RNA is detected in 2-cell embryos. (F) 28-cell embryo showing hybridization in all somatic cells. (D,H) No hybridization was observed in embryos hybridized with a *let-418* sense probe (compare D and C) and *chd-3* sense probe (compare H and G). *C. elegans* embryos are approximately 45 μm in length.

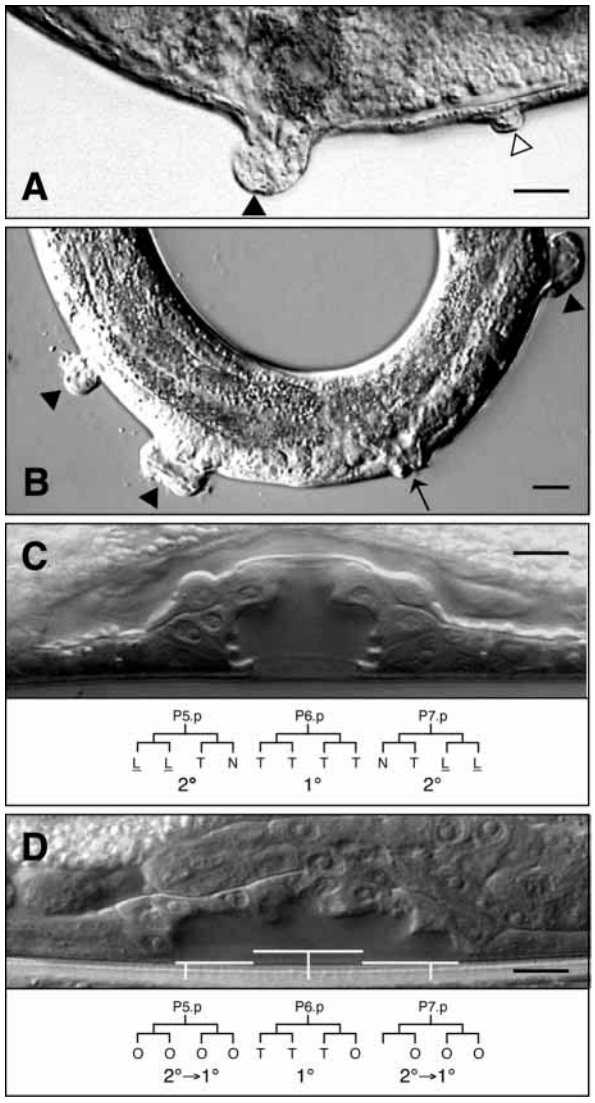

Fig. 3. Nomarski images of vulval phenotypes of *let-418* and *chd-3* hermaphrodites. (A) *let-418* adult showing an everted vulva (Evl) (closed arrowhead) and a posterior pseudovulva (open arrowhead) derived from an induced P8.p cell. (B) Synthetic Muv (synMuv) phenotype of *let-418(ar114);lin-15(n767)* double mutant showing multiple pseudovulvae derived from induction of P3.p, P4.p and P8.p (closed arrowheads, left to right). The normal vulva is indicated by an arrow. (C) Wild-type vulva in the late L4 stage. P5.p, P6.p and P7.p express secondary, primary and secondary cell fates, respectively. P(5-7).p cell lineages are indicated below using the nomenclature of Sternberg and Horvitz (1989); T, the nucleus divides transversally (left-right); L, the nucleus divides longitudinally (anterior-posterior); N, the nucleus does not divide. Underlining indicates that the daughter nuclei adhere to the cuticle (typical for the 2° fate). (D) vulva of a *let-418(ar114);chd-3(eh4)* double mutant from the same stage as in C. In this example, both P5.p and P7.p express an altered cell fate. Tissues derived from the P5.p, P6.p and P7.p cells are indicated by white lines. The P(5-7).p cell lineages are indicated below. O, cells divided obliquely. Anterior is left, posterior right. Scale bar, 10 μm.

not eliminated by backcrossing. In contrast, *let-418(ar114)*;synMuvB animals displayed no characteristic synMuv phenotype, though we noticed a slight increase in the frequency with which P8.p cells gave rise to a posterior pseudovulva relative to *let-418* single mutants (Table 2A and data not shown). RNAi experiments in which *lin-15A* (a class A gene) or *lin-15B* (a class B gene) dsRNA (Montgomery et al., 1998) was injected into a *let-418* mutant background, confirmed the above results (Table 2A). Epistasis analysis with *let-23(sy97)*, a mutation in the receptor tyrosine kinase encoding gene, showed that the *let-418; lin-15(n767)* synMuv phenotype requires a functional RTK/Ras pathway (Table 2A), as previously observed for other synMuv genes (Ferguson et al., 1987; Huang et al., 1994; Lu and Horvitz, 1998; Thomas and Horvitz, 1999). Based on these data, we conclude that *let-418* is a class B synMuv gene, but does not behave like a class A synMuv gene.

No Muv phenotype could be detected in either *chd-3*; class

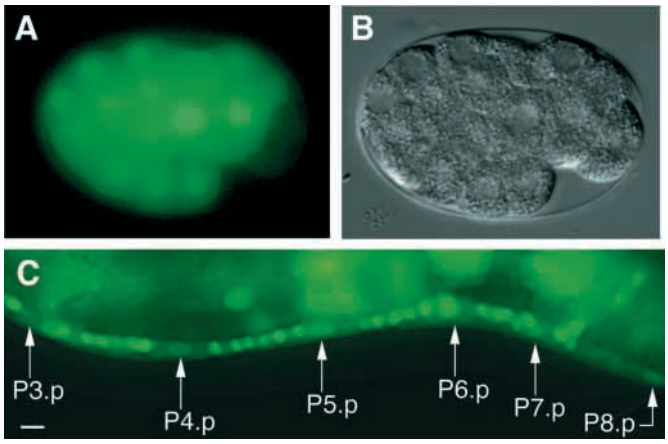

Fig. 4. *LET-418*::*GFP* reporter expression. (A) 28-cell embryo showing GFP expression in all somatic blastomeres. (B) Nomarski image of the same embryo as in A. (C) GFP is expressed in the nuclei of P(3-8).p cells (arrows, from left to right) of L3 animals. The adjacent and overlying stronger signals correspond to ventral nerve cord nuclei. Anterior is left, posterior right. Scale bar, 10 μm.

A (*lin-38;chd-3*, *lin-8;chd-3* or *chd-3;lin-15A*(RNAi)) or in *chd-3*; class B (*lin-9;chd-3* or *chd-3;lin-15B*(RNAi)) animals (data not shown). These results suggested that *chd-3* behaves neither like a synMuvA nor like a synMuvB gene. However, given the fact that *chd-3* and *let-418* have redundant functions during development, it remains possible that the two genes are also redundantly required for the synMuvA pathway. In such a model, *let-418* would possess both synMuvB and synMuvA activity, the latter being redundant with a synMuvA activity of *chd-3*. We have tested this model by constructing *chd-3;let-418* double mutants. With the exception of two cases of an induced P4.p cell (n=56), no significant ectopic induction of P3.p or P4.p cells, typical of synMuv animals, was found (Table 2B). 25% of *chd-3;let-418* animals did show a pseudovulva derived from P8.p, but this feature is inherent to *let-418* single mutants. We therefore conclude that *chd-3* and *let-418* are not redundantly required for the synMuvA pathway.

LET-418 and *CHD-3* are redundantly involved in the establishment of the 2º fate of P5.p and P7.p

Interestingly, by analyzing the phenotype of *let-418*; *chd-3* double mutants, we found that both proteins function in another cell fate determination event during vulval development. In *chd-3* or *let-418* single mutants, all P5.p and P7.p cells had the wild-type 2° cell fate, i.e. they generated one daughter that produces two laterally dividing cells adhering to the ventral cuticle and one daughter that produces one transversally dividing and one non-dividing cell that are both nonadhering to the cuticle (Fig. 3C). In *let-418; chd-3* double mutants, however, 30% of the P5.p and 40% of the P7.p cells produced four obliquely dividing cells that were all detached from the cuticle, resulting in the formation of an abnormal vulva (n=56) (Fig. 3D). The production of non-adhering cells is typical for the 1° sublineage, suggesting that P5.p and P7.p adopted a 1°-like or a 1°/2° hybrid cell fate (Sternberg and Horvitz, 1989). These data suggest that CHD-3 and LET-418 are redundantly involved in the proper specification or subsequent execution of the 2° fate that is established in response to *lin-12* Notch lateral signalling from P6.p (Greenwald et al., 1983).

DISCUSSION

Here we report the identification and genetic analysis of the two *C. elegans* Mi-2 homologues, *let-418* and *chd-3*. All three of the analyzed *let-418* alleles, *ar113*, *ar114* and *s1617*, contain premature stop codons and are strong loss-of-function or null alleles by genetic criteria. The only available mutation of *chd-3*, a deletion derivative that results in a severely truncated putative protein, is also likely to represent a strong loss-of-function or null allele. Using a genetic approach we show that *let-418*, but not *chd-3*, is an essential gene. During development, both genes have partially redundant activities, as *let-418*;*chd-3* double mutant animals are more severely affected than *let-418* single mutant animals.

In addition, the two genes are required for specific aspects of vulval development. We find that *let-418*, but not *chd-3*, behaves like a synMuvB gene. The incomplete penetrance of the Muv phenotype in synMuvA;*let-418* animals is likely to result from a partial maternal rescue of *let-418*, as reported for other synMuv genes (Huang et al., 1994). We have also tested whether *let-418* and *chd-3* function in the synMuvA pathway. However, none of the double mutant combinations synMuvB;*let-418*, synMuvB;*chd-3* or *let-418*;*chd-3* resulted in the formation of multiple pseudovulvae, showing that neither *let-418* nor *chd-3* behaves like a synMuvA gene. The finding that LET-418 acts in the synMuvB pathway, which includes products similar to the human retinoblastoma protein (LIN-35 Rb), the histone deacetylase 1 (HDA-1) and the Rb binding protein p48 (LIN-53 RbAp48) (Lu and Horvitz, 1998; Solari and Ahringer, 2000), can be incorporated into a previous model for transcriptional repression by the synMuv gene products (Lu and Horvitz, 1998). We postulate that a NuRD-like complex containing LET-418 Mi-2, recruited by LIN-35 Rb and sequence-specific transcription factors, is responsible for turning off vulval specification genes in the P3.p, P4.p and P8.p cells, perhaps by inducing a repressive chromatin structure. Recently, when the work presented in this paper was almost completed, a link between the synMuv genes and the NuRD complex was postulated based on protein interaction mapping (Walhout et al., 2000) and on RNAi experiments (Solari and Ahringer, 2000). Our results, however, do not fully support the RNAi based model proposed by Solari and Ahringer (2000), which suggests that both *C. elegans* Mi-2 proteins, LET-418 and CHD-3, function in the synMuvA and in the synMuvB pathways. The discrepancy between these and our genetic data may be explained by the following two observations: (1) *let-418* and *chd-3* RNAi may not act gene-specifically. We found evidence of cross-interference (results not shown), probably because of a very high DNA sequence similarity between the two genes. (2) Loss-of-function mutations in the synMuv genes result in the expression of ectopic vulval fates in all of the three cells, P3.p. P4.p and P8.p. Since induction of P8.p alone is a feature inherent of *let-418*, we only scored animals with multiple pseudovulvae as synMuv, whereas Solari and Ahringer also scored animals with a single pseudovulva (i.e. animals with two sites of vulval tissue; Solari and Ahringer, 2000).

We find that *chd-3* and *let-418* are redundantly involved in another vulval cell fate decision, namely in the proper specification or subsequent execution of the 2° fate in P5.p and P7.p that is established in response to *lin-12* Notch lateral signalling from P6.p. In *let-418*; *chd-3* double mutants, 30% of the P5.p and 40% of the P7.p adopt a 1°-like or a 1°/2° hybrid cell fate. It has been proposed that the 1° versus 2° cell fate decision in P(5,7).p takes place in VPCs during late G_1 or early S phase by acting in opposition to RTK/Ras inductive signalling to inhibit the 1° fate and permit the expression of the 2° fate (Ambros, 1999). We found that *let-418; chd-3* double mutants blocked in late VPC S phase never expressed *egl-17*::GFP, an early marker of the 1° fate (Burdine et al., 1998; Ambros, 1999) in either P5.p or P7.p (data not shown). Assuming that the timing of 2° fate specification is not altered, this may indicate that LET-418 and CHD-3 are not necessary for the early specification of the 2° versus 1° fate in these cells. Rather, they may act in a subsequent step required for the proper execution of the 2° fate, e.g. by maintaining the repression of 1° fate-specific genes. A maintenance function has been proposed for the *Drosophila* Mi-2 homologue, which genetically interacts with the Polycomb-group proteins to maintain the repression of *Hox* genes (Kehle et al., 1998). The *let-418*(*lf*); *chd-3*(*lf*) animals resemble the *lin-12*(*lf*) mutants, which lack 2º fates and show some ectopic induction of P4.p (Sternberg and Horvitz, 1989). Therefore, it is tempting to speculate that the two *C. elegans* Mi-2 proteins are linked to the LIN-12 Notch pathway. However, it is also possible that the primary defect in these animals lies not in the establishment of the 2° cell fate of P5.p and P7.p in response to LIN-12 Notch signalling, but rather in the increased sensitivity of all VPCs to RTK/Ras/MAP kinase signalling. In this context, it is interesting to note that P8.p is particularly sensitive to induction in both *let-418* single and *let-418*;*chd-3* double mutants (Table 2A). More experiments will be required to further explore the cause of the *let-418*; *chd-3* phenotype. Furthermore, whereas LET-418 may act together with other synMuv gene products as a component of a *C. elegans* NuRD complex, it is not clear whether CHD-3 is also part of a NuRD or NuRD-like complex. The human genome also encodes two closely related Mi-2 homologues, Mi-2α (HsCHD3) and Mi-2β (HsCHD4) (Woodage et al., 1997). Whereas Mi-2β is part of the human NuRD complex, preliminary evidence suggests that Mi-2α may exist in a complex different from NuRD (Xue et al., 1998).

In summary, our findings suggest that *C. elegans* Mi-2-dependent chromatin remodelling is involved in different cell fate decisions during vulval specification, and underline the importance of Mi-2 chromatin remodelling complexes during development. Our results give genetic support for a recently proposed link between the *C. elegans* Mi-2 homologues and the cancer-related Ras signalling pathway (Solari and Ahringer, 2000). This is of particular interest given the fact that the human Mi-2 proteins were originally identified as autoantigens in the human disease dermatomyositis, and patients with this disease face an increased risk of malignancy (Zhang et al., 1998). This opens the possibility that in human cells aberrant regulation of the Mi-2 proteins (and consequently of the NuRD or related complexes) may alter the expression of target genes regulated by the Ras (and perhaps the Notch) pathway, leading to metastatic growth potential.

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