NURD-complex genes antagonise Ras-induced vulval development in *Caenorhabditis elegans*

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Chromatin-modifying complexes are important for transcriptional control, but their roles in the regulation of development are poorly understood. Here, we show that components of the nucleosome remodelling and histone deacetylase (NURD) complex [1–5] antagonise vulval development, which is induced by the Ras signal transduction pathway. In three of the six equivalent vulval precursor cells, the Ras pathway is active, leading to the production of vulval fates [6]; in the remaining three, the Ras pathway is inhibited and vulval fates repressed. Inhibition of Ras signaling occurs in part through the action of the synthetic multivulval (synMuv) genes, which comprise two functionally redundant pathways (synMuvA and synMuvB) [7]. We found that five *Caenorhabditis elegans* members of the NURD chromatin remodelling complex inhibit vulval development through both the synMuvA and synMuvB pathways (*hda-1*, *rba-1*, *lin-53*, *chd-3* and *chd-4*); a further two members, the *MTA1*-related genes *egr-1* and *egl-27*, act only in the synMuvA pathway. We propose that the synMuvA and synMuvB pathways function redundantly to recruit or activate a core NURD complex, which then represses vulval developmental target genes by local histone deacetylation. These results emphasise the importance of chromatin regulation in developmental decisions. Furthermore, inhibition of Ras signaling suggests a possible link between NURD function and cancer.

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Results and discussion

We previously showed that *egr-1* and *egl-27* are redundantly required for embryonic patterning [8] and are similar to the human metastasis tumor associated gene *MTA1* [9]. We used RNA-mediated interference (RNAi) to study further the function of *egr-1*. Injection of an adult hermaphrodite with double-stranded (ds)RNA corresponding to a gene of interest will inactivate that gene in her progeny [10]. The *egr-1(RNAi)* animals appear essentially wild type at hatching [8], but at the adult stage they are sterile and form ectopic vulval tissue at low frequency (the multivulval or Muv phenotype; Table 1 and Figure 1b,c), suggesting that one function of *egr-1* is to repress vulval development, perhaps through interference with Ras signaling.

Animals mutant in a gene of either the synMuvA or synMuvB pathway or double mutants of genes in the same pathway have normal vulval development, but any synMuvA; synMuvB double mutant is Muv [7]. Two genes with similarity to components of chromatin-modifying complexes, *hda-1* (a histone deacetylase) and *lin-53* (a homologue of the retinoblastoma (Rb)-associated protein RbAp48), have been shown to act as synMuvB genes, but the mechanism by which the synMuvA pathway acts is not known [7,11]. Because EGR-1 is also a component of a chromatin-remodelling complex, we tested whether *egr-1* functioned in the synMuvB pathway. We found that *synMuvA*; *egr-1(RNAi)* double mutants do not show an increased frequency of Muv animals over *egr-1(RNAi)* in a wild-type background, indicating that *egr-1* does not function in the synMuvB pathway (Table 1). However, *egr-1(RNAi)*; *synMuvB* double mutants are Muv at high frequency (Table 1 and Figure 1e). Therefore, *egr-1* functions in the synMuvA pathway. This indicates that the synMuvA pathway, like the synMuvB pathway, has chromatin-remodelling functions.

In a mutant in which the Ras pathway is overactive because of a gain-of-function mutation in the *let-60* Ras gene, excess vulval tissue is made [12]. To further explore whether *egr-1* functions to antagonise Ras signaling, we asked whether loss of *egr-1* activity would enhance the Muv phenotype of *let-60(n1046gf)*. Indeed, *egr-1(RNAi)*; *let-60(n1046gf)* animals are significantly more Muv than *let-60 (n1046gf)* alone (92%, $n = 199$ versus 64%, $n = 283$). Injection of dsRNA of an unrelated gene (*vab-7*) had no effect on the Muv frequency (data not shown). These results indicate that *egr-1* has a role in repressing the Ras pathway.

We found that two alleles that were previously attributed to the complex locus *lin-40*, called *s1593* and *s1669*, introduce stop codons into the *egr-1* coding sequence (see Supplementary material). Analyses of *lin-40* alleles showed that they identify at least three different genes (F.S., B. Johnsen, D. Baillie and J.A., unpublished observations); *s1593* and *s1669* will now be called alleles of *egr-1*. Both *egr-1(s1593)* and *egr-1(s1669)* become sterile adults with abnormal gonads, similar to *egr-1(RNAi)*

Table 1

Components of the NURD complex have synMuvA and synMuvB properties.

Gene used for RNAi	Method used	Wild type	synMuvA		synMuvB	
			lin-15A	*lin-38*	*lin-37*	*lin-9*
None	None	**0%** (400)	**0%** (130)	**0%** (170)	**0%** (180)	**2%** (179)
egr-1	Inj	**3%** (0–5%, 254)	**2%** (0–5%, 176)	**3%** (0–5%, 140)	**61%** (50–79%, 61)	**36%** (14–55%, 138)
egr-1	Soak	**0%** (0–0%, 159)	**1%** (0–1%, 162)	–	**21%** (19–23%, 184)	–
egl-27	Soak	**<1%** (0–1%, 371)	**0%** (0–0%, 169)	–	**5%** (4–5%, 189)	–
egr-1 + egl-27	Soak	**0%** (0–0%, 184)	**0%** (0–0%, 98)	–	**50%** (50–50%, 135)	–
*lin-53**	Soak	**0%** (0–0%, 136)	**25%** (22–27%, 112)	–	**11%** (8–13%, 151)	–
*rba-1**	Soak	**0%** (0–0%, 145)	**2%** (2–2%, 129)	**1%** (76)	**19%** (15–21%, 157)	**17%** (41)
lin-53 + rba-1	Soak	**0%** (0–0%, 169)	**21%** (18–22%, 100)	–	**31%** (22–40%, 135)	–
*hda-1**	Soak	**6%** (4–9%, 139)	**12%** (3–22%, 168)	**12%** (12–13%, 163)	**53%** (52–65%, 506)	**40%** (34–50%, 91)
chd-3	Soak	**<1%**† (0–1%, 177)	**30%** (22–47%, 89)	**3%** (37)	**5%**§ (1–7%, 186)	**13%**§ (62)
chd-4	Soak	**0%**† (0–0%, 207)	**23%**‡ (15–32%, 128)	**1%** (69)	**6%**§ (3–8%, 164)	**15%**§ (86)
chd-3 + chd-4	Soak	**0%** (0–0%, 137)	**20%** (41)	**1%** (74)	**3%** (88)	**21%** (67)

Figures in bold are percentage Muv, with the range of results from multiple RNAi experiments as appropriate (up to 6 replicas) and the total number of animals scored in parentheses. Inj, RNAi was performed by injection; soak, RNAi was performed by soaking. Experiments were carried out at 22°C. Wild-type, synMuvA (*lin-15A* and *lin-38*) and synMuvB (*lin-37* and *lin-9*) headings give the genotypes used in the RNAi experiment. Dash, not done. The following alleles were used: *lin-15A(n767)*, *lin-38(n751)*, *lin-37(n758)*, and *lin-9(n112)* [7]. *Injection of dsRNAs to these genes results in embryonic lethality ([11,15] and data not shown). †Larval lethal when injected at 22°C; significantly Muv when injected at 15°C, suggesting an essential role in repressing vulval development. ‡After injection at 15°C, 50% of *chd-4(RNAi)*; *lin-15A* animals died. §Larval lethal when injected into these synMuvB backgrounds at 15°C.

animals (Figure 1f,g and data not shown). Expression of an *egr-1::gfp* (green fluorescent protein) reporter gene suggests that EGR-1 is a ubiquitous nuclear protein (see Supplementary material).

To confirm that *egr-1* acts in the synMuvA pathway, we constructed double mutants between *egr-1(s1593)* and either a *synMuvA* mutant (*lin-15A(n767)*) or a synMuvB mutant (*lin-37(n758)*). The *egr-1(s1593)* animals are Muv at low frequency (5%; $n = 100$), similar to *egr-1(RNAi)* (Table 1 and Figure 1f,g). We found that *egr-1(s1593)*; *lin-37* double mutants, but not *egr-1(s1593)*; *lin-15A* double mutants, are Muv at high frequency (39%, $n = 108$ versus 1%, $n = 109$; Figure 1h,j). Furthermore, *egr-1(s1593)*; *lin-15B(RNAi)* are often Muv (26%, $n = 53$; *lin-15B* is a synMuvB gene) whereas *egr-1(s1593)*; *lin-15A(RNAi)* are not (0%, $n = 43$). Therefore, the RNAi and mutant data both indicate that *egr-1* is a synMuvA gene.

Because *egr-1* and *egl-27* are functionally redundant in embryonic patterning [8], we investigated whether *egl-27* also has a role in vulval development. On its own, *egl-27* does not have a strong interaction with *synMuvA* or *synMuvB* genes (Table 1). However, *egr-1(RNAi)*; *egl-27(RNAi)*; *synMuvB* triple mutants show a large increase in the percentage of Muv animals over *egr-1(RNAi)*; *synMuvB* double mutants (Table 1). This shows that *egl-27* and *egr-1* are partially redundant in vulval development, although *egr-1* appears to be more important.

EGR-1 and EGL-27 are similar to one component of the NURD complex, Mta1. We next explored other *C. elegans* members of the NURD complex for roles in vulval development. Besides MTA1, the NURD complex contains HDAC1 and HDAC2 (histone deacetylases), RbAp46/48 (Rb-associated proteins), CHD3 and/or CHD4 (highly similar chromodomain helicase proteins), MDB3 (similar to methyl-CpG-binding proteins) and several uncharacterised polypeptides [1–5,13]. In *C. elegans*, two genes encoding RbAp46/48 homologues, *rba-1* and *lin-53* (previously known as *rba-2*), and three histone deacetylases, *hda-1*, *hda-2* and *hda-3*, have been previously described [11,14]. In a search of the current *C. elegans* genomic sequence (over 99% complete), we identified two good matches to CHD3 and CHD4 (T14G8.1 and F26F12.7); each is 48% identical to both human CHD3 and CHD4, and we have named the corresponding genes *chd-3* and *chd-4*, respectively. At present, there is no clear *C. elegans* match to MDB3.

Previous work showed that *hda-1* and the RbAp46/48 gene *lin-53* are synMuvB genes [11]. Both are predicted to encode proteins in the NURD complex. However, we found that *egr-1*, encoding one member of this complex, is a

synMuvA gene. To address this conundrum, we performed RNAi with *hda-1* and *lin-53* in wild-type, synMuvA and synMuvB backgrounds. Injection of dsRNA corresponding to either gene causes embryonic lethality ([8] and data not shown). To overcome this technical difficulty, we performed the RNAi experiments by soaking first-stage larvae in dsRNA; this has been shown to be an effective alternative method to injection of mothers with dsRNA, although it produces a slightly weaker effect [15].

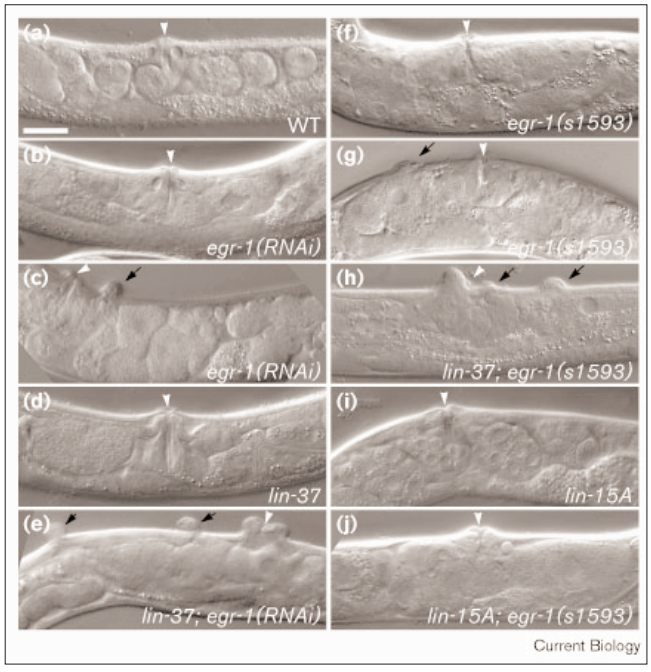
Surprisingly, *hda-1(RNAi)* and *lin-53(RNAi)* have both synMuvA and synMuvB properties (Table 1). For example, as previously reported [11], *lin-53* behaves as a synMuvB gene, because *lin-53(RNAi)* produces Muv animals in a synMuvA background but not in wild-type animals (Table 1). However, *lin-53(RNAi)*; synMuvB animals are also Muv (Table 1), indicating that *lin-53* is also a synMuvA gene. The other RbAb46/48 homologue, *rba-1*, also has both synMuvA and synMuvB properties (Table 1). The genes do not appear to be redundant, as the frequency of Muvs is only additive (and not greater as would be expected for redundant genes) when *rba-1* and *lin-53* are inactivated together. These results suggest that each of the two *C. elegans* RbAp46/48 homologues functions in both synMuvA and synMuvB pathways.

The *hda-1* gene was previously shown to have synMuvB characteristics, but was not tested for synMuvA activity [11]. We found that *hda-1(RNAi)*; synMuvA animals are Muv (Table 1), confirming that *hda-1* is a synMuvB gene. In a synMuvB background, *hda-1(RNAi)* also produces Muv animals at high frequency (Table 1). Therefore, like the RpAp48 homologs, *hda-1* appears to act in both pathways. Neither *hda-2(RNAi)* nor *hda-3(RNAi)*, nor *RNAi* to both together, produced Muv animals in any background (data not shown and [14]).

Finally, we tested whether *chd-3* and *chd-4* act in either synMuv pathway: *chd-3(RNAi)* and *chd-4(RNAi)* each produces a significant percentage of Muvs in both synMuvA and synMuvB mutant backgrounds (Table 1), indicating that *chd-3* and *chd-4* act in both pathways. The *chd-3* and *chd-4* genes appear to have non-redundant functions, because removing both together does not increase the frequency of Muvs (Table 1).

In summary, we have shown that members of the NURD chromatin-remodelling complex inhibit vulval development within both the synMuvA and synMuvB pathways. Biochemical analyses in a number of systems have identified a NURD complex with the composition described above [1–5,13]. The simplest interpretation of our finding of the involvement of each NURD member in repression of vulval development is that these proteins act together in a single complex, but it is possible that individual members might have independent functions in the vulva as well.

Figure 1

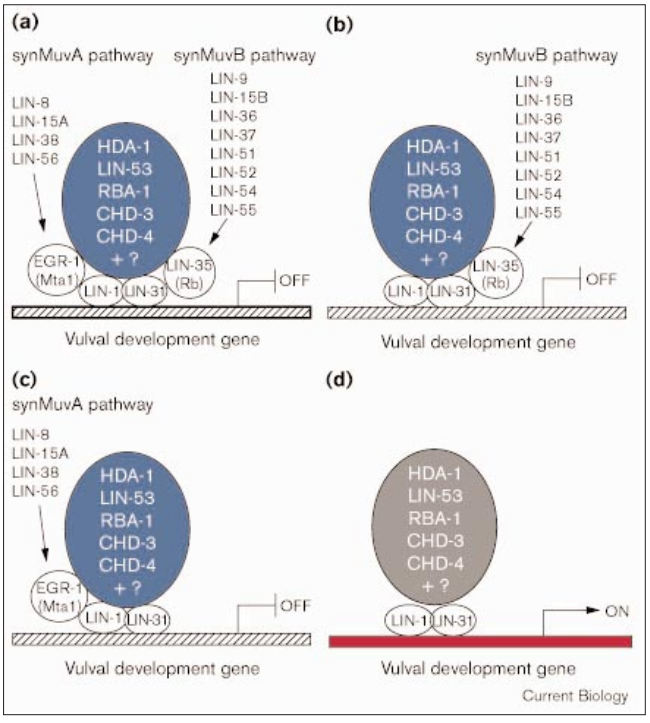


Vulval phenotypes induced by *egr-1(RNAi)* and *egr-1(s1593)*. Genotypes are given in the figure. **(a,b,d,f,i,j)** show normal vulvae; **(c,e,g,h)** have ectopic vulval tissue. Both phenotypes were seen in *egr-1(RNAi)* and *egr-1(s1593)* animals. White arrowheads indicate the central vulva; black arrows indicate ectopic vulval tissue. The scale bar represents 50 μm.

Complexes such as NURD are proposed to interact with sequence-specific transcription factors, resulting in chromatin changes and repression of target genes [1,3–5]. We propose that the redundant synMuvA and synMuvB pathways activate or recruit a core NURD complex (containing HDA-1, RBA-1, LIN-53, CHD-3, CHD-4, and possibly other proteins), resulting in repression of vulval development genes (Figure 2). Interaction of the core NURD complex with either the synMuvA or synMuvB pathway would be sufficient for vulva-specific activity. Unexpectedly, the two MTA1 homologues, EGR-1 and EGL-27, which were thought to be integral to the NURD complex, appear to act only in the synMuvA pathway. This raises the possibility that EGR-1 and EGL-27 may function within the synMuvA pathway to recruit a partial NURD complex. Likewise, the Rb-like gene *lin-35* functions only in the synMuvB pathway [11]. Rb is a transcriptional corepressor, and recent biochemical studies have linked Rb to the histone deacetylase HDAC1 and a CHD3/4-like protein Mi2β [16–19]. Therefore, LIN-35 could be a synMuvB adapter. Possible transcription factor targets of the complex are LIN-1 and LIN-31, which are vulval development repressors regulated by Ras signaling [20].

Previous screens for vulval development genes failed to identify most of the genes we describe here. A likely

Figure 2



Model for regulation of vulval development genes by the NURD complex. **(a)** We propose that redundant synMuvA and synMuvB pathways function to recruit or activate a core NURD complex (blue oval) by direct interaction with EGR-1 or LIN-35. The NURD complex could then facilitate repression of vulval development genes by transcription factors inhibited by Ras signaling, perhaps a LIN-1–LIN-31 dimer [20], by deacetylating chromatin locally (OFF; hatched box). In the absence of either **(b)** synMuvA or **(c)** synMuvB pathway function, the complex could still be recruited and be functional. **(d)** When both pathways are non-functional, the NURD complex cannot modify chromatin of vulval development genes (red), leading to their activation (ON).

reason for this is that mutation of any of these genes would result in either a sterile or a lethal mutant phenotype. This indicates that NURD and/or other complexes containing these proteins have developmental roles in addition to those in the vulva. Furthermore, involvement of the NURD complex with the Ras signaling pathway could shed light on the connection of Ras with the cancerous state [21], as MTA1 expression correlates with metastatic potential of several human cancer cell lines [9] and aberrant regulation of histone deacetylase has been proposed to play a role in malignant transformation [22,23].

Supplementary material

Supplementary material including the *egr-1* sequence, data showing the *egr-1::gfp* expression pattern, controls showing the effectiveness of RNAi in the vulva and additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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