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XNP-1/ATR-X acts with RB, HP1 and the NuRD complex during larval development in *C. elegans*

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Abstract

Mutations in the *XNP/ATR-X* gene cause several X-linked mental retardation syndromes in humans. The *XNP/ATR-X* gene encodes a DNA-helicase belonging to the SNF2 family. It has been proposed that XNP/ATR-X might be involved in chromatin remodelling. The lack of a mouse model for the ATR-X syndrome has, however, hampered functional studies of XNP/ATR-X. *C. elegans* possesses one homolog of the *XNP/ATR-X* gene, named *xnp-1*. By analysing a deletion mutant, we show that *xnp-1* is required for the development of the embryo and the somatic gonad. Moreover, we show that abrogation of *xnp-1* function in combination with inactivation of genes of the NuRD complex, as well as *lin-35*/Rb and *hpl-2*/HP1 leads to a stereotyped block of larval development with a cessation of growth but not of cell division. We also demonstrate a specific function for *xnp-1* together with *lin-35* or *hpl-2* in the control of transgene expression, a process known to be dependent on chromatin remodelling. This study thus demonstrates that in vivo XNP-1 acts in association with RB, HP1 and the NuRD complex during development.

Keywords: ATR-X syndrome; Larval arrest; Chromatin remodelling; SWI/SNF complex; SynMuv genes

Introduction

Mutations in the human *XNP/ATR-X* gene are associated with several X-linked mental retardation syndromes, the best known being α-thalassemia with mental retardation (ATR-X). Patients are affected by a severe brain dysfunction, craniofacial abnormalities and can present an impaired urogenital system development (Gibbons and Higgs, 2000). Although the precise function of XNP/ATR-X remains unclear, several pieces of evidence suggest that it is involved in gene expression regulation via chromatin remodelling. First, it contains a SNF2-type ATP-dependent helicase motif found in proteins involved in chromatin remodelling, including the Mi-2 subunit of the nucleosome remodelling and histone deacetylase (NuRD) complex (Xue et al., 1998). Second, XNP/ATR-X has been found at pericentromeric heterochromatin (McDowell et al., 1999) and has been shown to interact with the heterochromatin protein HP1 and the polycomb protein EZH2 (Berube et al., 2000; Cardoso et al., 1998). More recently, it has been shown that XNP/ATR-X can be co-immunoprecipitated from HeLa cell extracts with the transcription cofactor Daxx in a complex that has several ATP-dependent activities consistent with it being a chromatin-remodelling complex (Xue et al., 2003).

We chose *Caenorhabditis elegans* as a simple model to investigate the in vivo function of XNP/ATR-X. The *C. elegans* homologue, XNP-1, is a protein of 1359 amino acids with approximately 45% identity to the human protein within the helicase domain (Villard et al., 1999). In this study, we demonstrate that the *xnp-1* gene is essential for *C. elegans* embryogenesis and gonadogenesis.

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We show that *xnp-1* genetically interacts with components of the NuRD complex, as well as with the *lin-35*/retinoblastoma (Rb) and *hpl-2*/HP1 genes. Together, these genes govern the progression of larval development. Moreover, we show that *xnp-1* enhances *lin-35*-dependent transgene silencing.

Materials and methods

Strains

All strains including the wild type N2 Bristol, IG256 *xnp-1(tm678)* (outcrossed 3× with N2 worms), JA1194 *egl-27(we3)* (Solari et al., 1999), MT556 *lin-9(n112) dpy-17(e164)* (Horvitz and Sulston, 1980), MT1806 *lin-15(n767)*, MT3033 *lin-35(n745) unc-13(e1091)*, MT1799 *lin-36(n766) unc-32(e189)*, MT5470 *lin-37(n758)*, MT1808 *lin-38(n751)* (Ferguson and Horvitz, 1989), MT8840 *lin-53(n833) dpy-5(e61)* (Lu and Horvitz, 1998), GS402 *let-418(ar114)/unc-46(e177) dpy-11(e224)* (Seydoux et al., 1993), PK168 crEx37[*sur-5*::GFP,*unc-119*] (a gift from P. Kuwabara), BK48 Ex*let-858*::GFP (Kelly et al., 1997), UG42 bgEx21[pB*unc-53*::GFP,*rol-6(su1006)*] (Stringham et al., 2002), JK2868 qIs56[*lag-2*::GFP,*unc-119*] (Henderson et al., 1994), IG261 frEx66[p*nlp-29*::GFP,p*col-12*::DsRed] (Couillault et al., 2004) and IG275 frIs9[p*nlp-29*::GFP,p*col-12*::DsRed] were grown and maintained at 20°C or 25°C as described (Brenner, 1974).

Reporter gene and RNAi constructs

The p*xnp-1*::GFP reporter contains 2572 bp of genomic sequence upstream of the start codon of *xnp-1* and 150 bp downstream. It was obtained by PCR amplification and cloned into pPD95.75 (a gift from A. Fire). Transgenic animals were generated using standard techniques (Mello et al., 1991). Two independent lines IG53 and IG54 were generated and had the same expression pattern. An RNAi vector insert targeting exons 5, 6 and 7 of *xnp-1* was constructed containing 930 bp of cDNA sequence excised with *Eco*RI from the clone pGEM-T/yk249d10 (a gift from L. Villard) and cloned into pPD129.36 (a gift from A. Fire). The resulting plasmid was used to transform the bacterial strain HT115(DE3). Control experiments used the same strain transformed with pPD129.36.

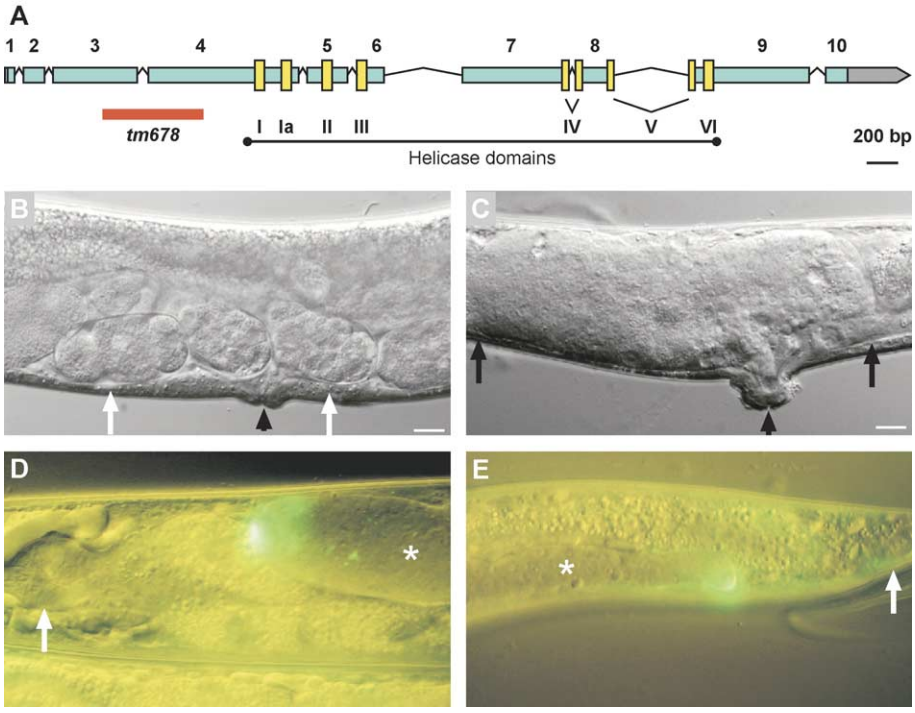

Fig. 1. (A) Structure of the *xnp-1* gene. Exons are shown as blue rectangles, introns as lines and the 3′ untranslated region in grey. The deletion in the *xnp-1(tm678)* allele (red bar) is upstream of all seven helicase domains (yellow boxes). The sequence is available in the public database Wormbase. (B–C) Gonadogenesis defect in the *xnp-1* mutant. Nomarski images of wild type (B) and *xnp-1* mutant (C) adults. In the wild type, fertilised eggs (white arrows) that will be laid via the vulva (black arrow) are visible. The two gonad arms extend anteriorly and posteriorly beyond the field of view. In the mutant, the gonad is abnormally small, its extent delimitated by the arrows, with an accumulation of somatic gonadal and germline cells in the immediate vicinity of an abnormally protruding vulva (arrowhead). (D–E) Overlay of Nomarski and fluorescent images of *xnp-1;lag-2*::GFP adult worms. The distal tip cell that expresses the reporter gene is fluorescent, often failed to perform the usual U-turn and continued to migrate anteriorly towards the pharynx or posteriorly towards the anus (arrows in D and E, respectively), the gonad is marked with an asterisk. Scale bar, 10 μm.

RNAi

L4 worms were placed at 20°C or 25°C on NGM agar, with IPTG and carbenicillin, spread with the appropriate RNAi bacterial strain (Kamath et al., 2001). The next day, adults were transferred to fresh plates and left to lay eggs for 2.5 h before being removed. The following days, the number of unhatched eggs was counted, and the development of the progeny observed. Each experiment was repeated at least three times. To test the effect of RNAi at the L1 stage, we grew worms on control RNAi plates as above but transferred their progeny at the L1 stage to plates spread with the appropriate RNAi bacterial strain. Injection of *xnp-1* dsRNA, or GFP dsRNA as a control, into the gonad of wild type adult worms was performed as previously described (Fire et al., 1998).

Results

xnp-1 is required for embryogenesis and somatic gonad development

The mutant allele *xnp-1(tm678)* contains a genomic deletion of 673 bp, between exons 3 and 4. We found by RT-PCR the expected altered *xnp-1* transcript in the mutant (data not shown). Since the lesion provokes a frame shift, introducing a stop codon in the 4th exon, *xnp-1(tm678)* is predicted to encode a truncated protein of 207 amino acids, lacking all seven conserved helicase domains, and is therefore likely to be a null allele (Fig. 1A).

Analysis of the mutants revealed *xnp-1* to be essential for embryogenesis at higher temperatures. The incidence of embryonic lethality in *xnp-1(tm678)* worms at 20°C was very low (3%; n = 804), but increased significantly at 25°C (38%; n = 385). Worms that successfully completed embryogenesis developed into adults, a large proportion of which were sterile (48% at 20°C; n = 40; 88% at 25°C; n = 40). We found that gonadogenesis was severely affected in the *xnp-1* mutant at 25°C. While in wild-type animals, the two gonad arms form an elongated U-shape tube, in *xnp-1* mutants they failed to develop correctly. In the most extreme cases, there was no gonadal growth and germline cells accumulated around the vulva (Figs. 1C and 4A). In other cases, there was a defect in gonad elongation or migration. In some individuals, the gonad arms stopped their growth before the position where in wild-type animals they would turn, while in others, they failed to turn and the distal tip cell continued its migration anteriorly or posteriorly, such that the tip of the gonads were found close to the pharynx or the anus, respectively (Figs. 1D, E). The distal tip cells appeared to be properly differentiated, as judged by the expression the *lag-2*::GFP reporter gene. A quarter of *xnp-1* mutants grown at 25°C had a protruding vulva (Pvl) phenotype (24%, n = 764; Fig. 1C), and occasionally had one pseudovulva (Muv; 0.2%, n = 764). Mutation of *xnp-1* was also associated with a high incidence of males (2%, n = 764). Injection of *xnp-1* dsRNA in wild type adults at 25°C gave rise to the same phenotypes in their progeny (data not shown). These results taken together with the RNA interference (RNAi) experiments described below and its molecular identity indicates that the *xnp-1(tm678)* mutant allele is a null.

Table 1
RNAi of *xnp-1* on SynMuv mutants[a]

	Mutant strain	Human homologue	Embryonic lethality (%)		Larval arrest (%)[b]		*n*	
			Control	*xnp-1*[c]	Control	*xnp-1*	Control	*xnp-1*
SynMuvA	*lin-15(n767)*	Novel	0.4	0.25	0	0	1233	1188
	lin-38(n751)	Not cloned	1	0.6	0	0	672	666
	egl-27(we3)	MTA-1	0.6	0.6	0	0	945	952
SynMuvB	*lin-9(n112)*	TGS	10.5	12.5	0	87	1002	950
	lin-35(n745)	Rb	0.1	0.6	21	95	792	706
	lin-36(n766)	Novel	0.4	0.2	3.9	26.5	539	580
	lin-37(n758)	Novel	1.2	0.8	10	97.7	764	859
	lin-53(n833)	RbAP46/48	2.3	1.6	12	98	463	467
	let-418(ar114)	Mi-2/CHD4	0.4[d]	0.8[d]	0[e]	99[e]	–	–

[a] All RNAi experiments were performed at both 25 and 20°C. The majority of the mutants failed to give a synthetic phenotype at 20°C when treated with *xnp-1* RNAi, with the exception of *lin-37* (85% of larval lethality (n = 680) against 0.5% (n = 674) for the control RNAi). The results presented were obtained at 25°C.

[b] The percentage shown excludes dead embryos.

[c] Contrary to the result of its injection, the ingestion of *xnp-1* dsRNA was never associated with a significant increase in embryonic lethality, probably due to the previously reported refractory nature of certain types of cell to this technique (Timmons et al., 2001).

[d] The percentage of dead embryos among the total progeny of heterozygous *let-418(ar114)/unc-46(e177) dpy-11(e224)* worms; control n = 289 and *xnp-1* n = 263.

[e] The percentage of arrested larvae among homozygous *let-418(ar114)* worms assuming that there were as many as *unc-46(e177) dpy-11(e224)* worms; control n = 36 and *xnp-1* n = 32.

Table 2
RNAi of SynMuv genes on the *xnp-1* mutant and wild type at 25°C

	RNAi	Human homologue	*xnp-1(tm678)*			Wild type	
			Larval arrest[a] (%)	Embryonic lethality (%)	*n*	Embryonic lethality (%)	*n*
	control	–	0	17[b]	623	1	825
	xnp-1	XNP/ATR-X	0	23	728	1[c]	1179
SynMuvA	*egl-27*	MTA-1	0	11	853	1	593
SynMuvB[d]	*lin-35*	RB	100	27	696	1	669
	hpl-2	HP1	100	17	482	1	753
	lin-37	Novel	98	11	605	4	758
	lin-9	Novel	94	21	365	0	682
	lin-36	Novel	0	16	295	0	609
	hda-1	HDAC1	0	91[e]	725	100[e]	1024

[a] No larval arrest was observed in the experiments with wild type worms. The percentage shown excludes dead embryos.
[b] The *xnp-1* worms were raised at 20°C and shifted to 25°C at the L4 stage, explaining why the percentage of embryonic lethality is lower than when the strain is maintained at 25°C; see text.
[c] The *xnp-1* RNAi did not give any phenotype with wild type worms, except occasionally Pvl adults, probably due to the previously reported refractory nature of certain types of cell to this technique (Timmons et al., 2001).
[d] *lin-53* and *let-418* were not tested as RNAi of these genes is lethal.
[e] Highly significantly different $P < 0.00001$ (Fisher test).

xnp-1 is strongly expressed in dividing cells

Northern analysis revealed a single transcript of the expected size (4.3 kb), consistent with the absence of alternative splice forms in publicly available cDNA sequence databases and our RT-PCR analyses (data not shown). *xnp-1* was present at a high level in eggs, and decreased in successive stages (Fig. 2A). In situ hybridisation revealed expression in the embryo and in the germ line (Fig. 2B; Y. Kohara, personal communication). To analyse further the expression of *xnp-1*, we generated transgenic worms carrying a reporter gene construct containing the 2.5-kb upstream genomic sequence of *xnp-1* fused in frame to the coding sequence of GFP. The reporter gene was strongly expressed, starting at mid-embryogenesis, when it was expressed almost ubiquitously (Figs. 3A–D). Subsequently, expression faded in most cells during embryonic morphogenesis. After hatching, strong expression was observed in all dividing cells, including at the first larval stage the P lineage that gives rise to adult motoneurones in the ventral cord (Figs. 3E–G) and in later larval stages in vulval precursor cells (data not shown). No expression was observed in the germ line, presumably due to transgene silencing in this tissue (Kelly et al., 1997).

Synthetic larval arrest between xnp-1 and NuRD complex mutants

In mammals *XNP/ATR-X* is thought to be involved in chromatin remodelling, so we inactivated *xnp-1* in combination with genes known to be involved in chromatin remodelling, in particular genes of the NuRD complex. In *C. elegans*, NuRD inhibits vulval development through the action of the Synthetic multivulva (SynMuv) genes that form two functionally redundant pathways: SynMuvA and SynMuvB. Animals mutant in a gene of either pathway or double mutants of genes in the same pathway have normal vulval development. In contrast, SynMuvA/SynMuvB double mutants display a Muv phenotype, which results from extra cells adopting the vulval fate (Ferguson and Horvitz, 1989). Some components of the NuRD complex act in the SynMuvB pathway, for example, LIN-53, a protein homologous to the mammalian RB-associated 46–48 kDa protein, HDA-1, a histone deacetylase, and LET-

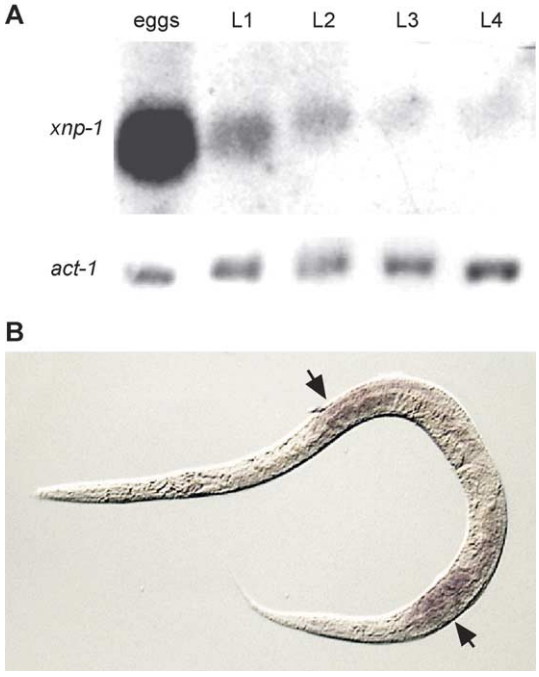

Fig. 2. Developmental expression of *xnp-1*. (A) Northern blot analysis of *xnp-1* expression was carried out as previously described (Villard et al., 1999). Upper panel: RNA prepared at different developmental stages was hybridised with a cDNA probe corresponding to *xnp-1*. Lower panel: the blot was stripped and rehybridised with an actin loading control, *act-1*. High expression is observed in eggs and decreases in successive developmental stages. (B) Whole mount in situ hybridisation analysis of *xnp-1* in a young adult worm. Data kindly provided by Yuji Kohara for the yk553g5 cDNA. Expression is observed in the germline (arrows).

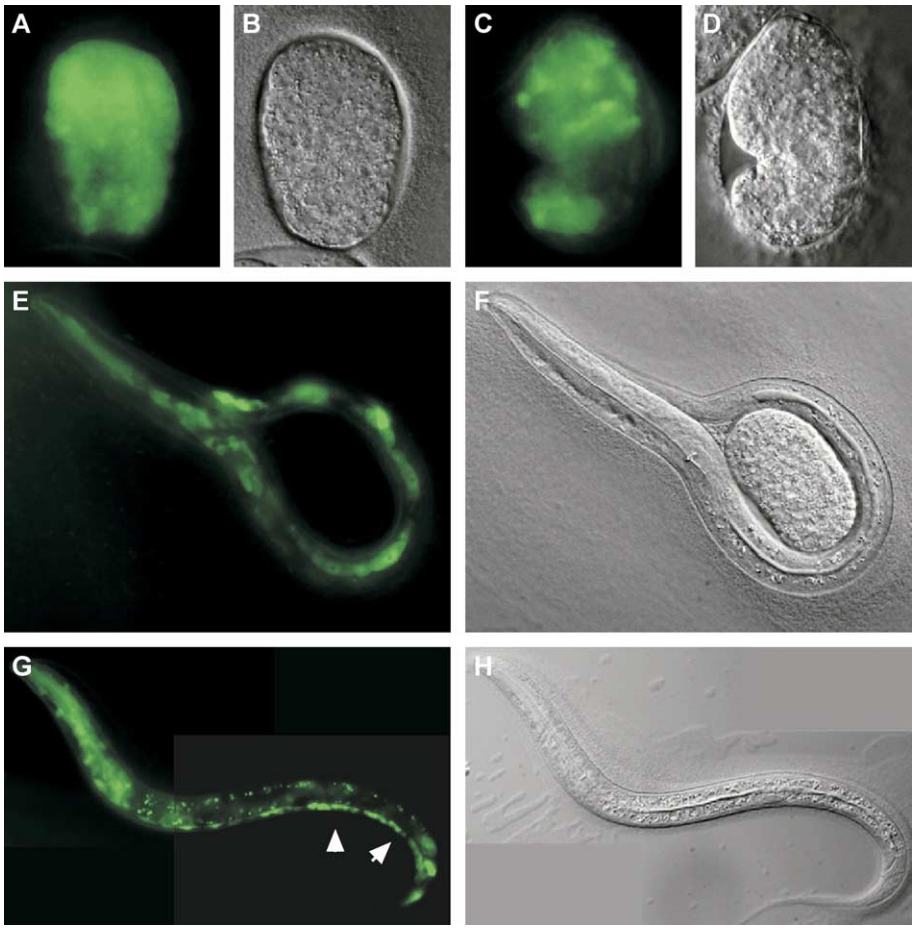

Fig. 3. Spatial and temporal expression of an *xnp-1*::GFP reporter construct. Fluorescent (A, C, E, G) and Nomarski (B, D, F, H) images showing that expression is almost ubiquitous in the late gastrula (A–B), more spatially restricted in the comma stage embryo (C–D), and high in dividing cells in L1 larvae (E–H), in the seam cells (E) and in the newly born motoneurones (G, arrowheads).

418, the *C. elegans* Mi-2 homologue. Others act in the SynMuvA pathway, like EGR-1 and EGL-27 proteins similar to metastasis tumour associated protein 1 (reviewed in Solari and Ahringer, 2000).

We examined the effect of RNAi (Fire et al., 1998) of *xnp-1* in different mutants of the SynMuvA and B pathways, and conversely of inactivation of the latter genes by RNAi in the *xnp-1* mutant. Strikingly, several SynMuvB but no SynMuvA mutants gave rise to a synthetic lethal phenotype when combined with abrogation of *xnp-1* function at 25°C. Thus, RNAi of *xnp-1* in a *lin-35(n745)*, *lin-53(n833), let-418(ar114), lin-9(n112)* or *lin-37(n758)* background gave a highly penetrant arrest at the first larval (L1) stage (Table 1). Conversely, RNAi of *lin-35*, *lin-9* or *lin-37* in the *xnp-1(tm678)* background gave an almost completely penetrant L1 arrest at 25°C (Table 2, Fig. 4B).

One of the *C. elegans* HP1 homologues, *hpl-2*, has been shown to act as a SynMuvB gene. Interestingly, injection of *hpl-2* dsRNAs in *lin-9(n112), lin-35(n745)* or *lin-37(n758)* mutants also provokes larval lethality (Couteau et al., 2002). In mammals, HP1 is a partner of the XNP/ATR-X protein (Berube et al., 2000). When we inactivated *hpl-2* by RNAi in the *xnp-1* mutant background at 25°C, 100% of the hatching embryos died as L1 larvae (Table 2, Fig. 4C), with the same phenotypes as observed when *lin-35*/Rb and *xnp-1* were inactivated. These L1 larvae continued feeding but their size remained that of young L1 larvae (Fig. 4D) until their death after 2 to 7 days.

One known class of mutants exhibiting L1 larval arrest is the *lag* (*lin-12* and *glp-1*) mutants that also exhibit characteristic defects in excretory canal and anal development (Lambie and Kimble, 1991). The *xnp-1(tm678)*;*lin-35*(RNAi) arrested larvae did not resemble the *lag* mutants as both the excretory canal and anus were correctly formed (data not shown). To characterise further the nature of the arrested state, we examined different tissues in these larvae using Normarski microscopy and fluorescent reporter genes. With regards to the gonad, the arrested larvae had either the primordium composed of the two somatic gonad (Z1 and Z4) and the two germline (Z2 and Z3) precursor cells seen normally in young L1 larvae (Figs. 4C and 5C), or a dozen cells, arising from the three rounds of cell division of the somatic gonad that normally occur before the end of L1 stage (Kimble and Hirsh, 1979). The *lag-2*::GFP reporter gene, known to be present in Z1 and Z4 in the L1 stage

(Henderson et al., 1994), was correctly expressed (see Supplementary information). Using an *unc-53*::GFP reporter (Stringham et al., 2002), we observed that the post-embryonic AS motoneurones that are born from the P cells and migrate to the ventral cord at the late L1 stage in wild-type worms (Sulston and Horvitz, 1977) were present in the arrested larvae (Fig. 5D). Using the same reporter, we observed that the plasmid socket cells arising from the postembryonic division of the T cell in the mid L1 stage (Sulston and Horvitz, 1977) were present, started their differentiation, but do not grow (results not shown). The seam cells, which do not normally divide before the L1–L2 transition, when they undergo two rounds of division (Sulston and Horvitz, 1977), were found to divide, so that arrested individuals, the size of young L1 larvae, had the number of seam cells usually found in young L2 larvae (Fig. 5H). In summary, these data show that a subgroup of SynMuvB genes, including some of the NuRD complex, *lin-35* and *hpl-2* genetically interacts with *xnp-1* during *C. elegans* larval development. Furthermore, this synthetic arrest appears to be associated with a cessation of growth but not of cell division.

The precocious death of the SynMuvB/*xnp-1* mutant worms prevented the potential role of *xnp-1* in the formation of the vulva from being assayed. In an attempt to circumvent this, we treated *lin-35*/Rb mutants with *xnp-1* RNAi, and conversely *xnp-1* mutants with *lin-35* or *hpl-2* RNAi, starting at the L1 stage. In all cases, development proceeded normally and no obvious synthetic defect was observed, suggesting, if larval RNAi is efficient, that *xnp-1* does not act as a classical SynMuv gene.

Synthetic larval arrest is associated with a decrease of sur-5::*GFP transgene expression*

While characterising the synthetic arrest phenotype described above, using reporter transgenes, we observed a marked decreased in the GFP expression of the ubiquitously expressed *sur-5*::GFP transgene in *xnp-1;lin-35* (RNAi) and *xnp-1;hpl-2*(RNAi) arrested larvae. The *sur-5*::GFP signal totally disappeared from the hypodermis and intestinal cells, but remained in the nervous system and some pharyngeal cells, presumably reflecting the refractory nature of these cells to RNAi by feeding (Timmons et al., 2001), (Fig. 6 and results not shown). Expression of a second ubiquitous reporter transgene *let-858*::GFP was not affected by the combined inactivation of *xnp-1* and *lin-35*, nor was that of a *unc-53*::GFP reporter nor that of the hypodermis-specific p*nlp-*

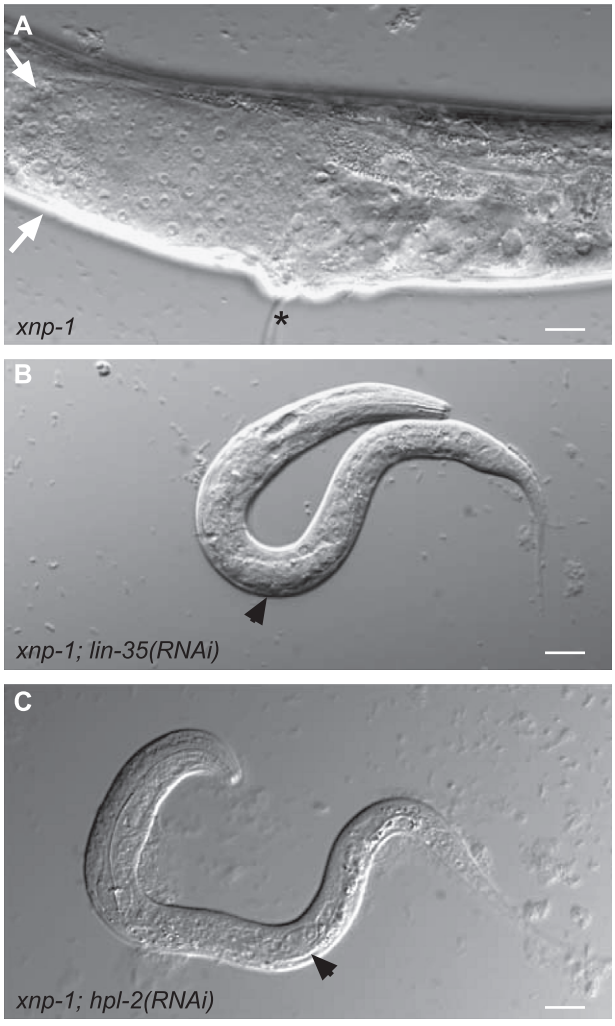

Fig. 4. Developmental arrest in *xnp-1(tm678)* mutants upon RNAi of *lin-35* or *hpl-2*. Nomarski images at the same magnification of *xnp-1* mutant animals 3 days after being laid at 25°C, following control RNAi (A), *lin-35* RNAi (B) or *hpl-2* RNAi (C). (A) The control animal is at the adult stage, with a clearly visible vulva (asterisk) but with a gross defect in gonadogenesis (the limit of the anterior gonad is indicated by arrows). (B, C) Inactivation of *lin-35* and *hpl-2* in *xnp-1(tm678)* worms provokes an L1 arrest. The gonad primordium is indicated by an arrowhead. Scale bar, 10 μm. (D) Quantification of the size of wild type or *xnp-1* mutant animals 48 h after being laid at 25°C, following control RNAi (white bars), *lin-35* RNAi (grey) or *hpl-2* RNAi (black). The graph shows the average time of flight (TOF) of the indicated number of worms under each experimental condition measured using the COPAS biosort. TOF is a measure of length, as previously detailed (Couillault et al., 2004). In all cases, the standard error of the mean (SEM) was less than 5% of the mean value and is not shown.

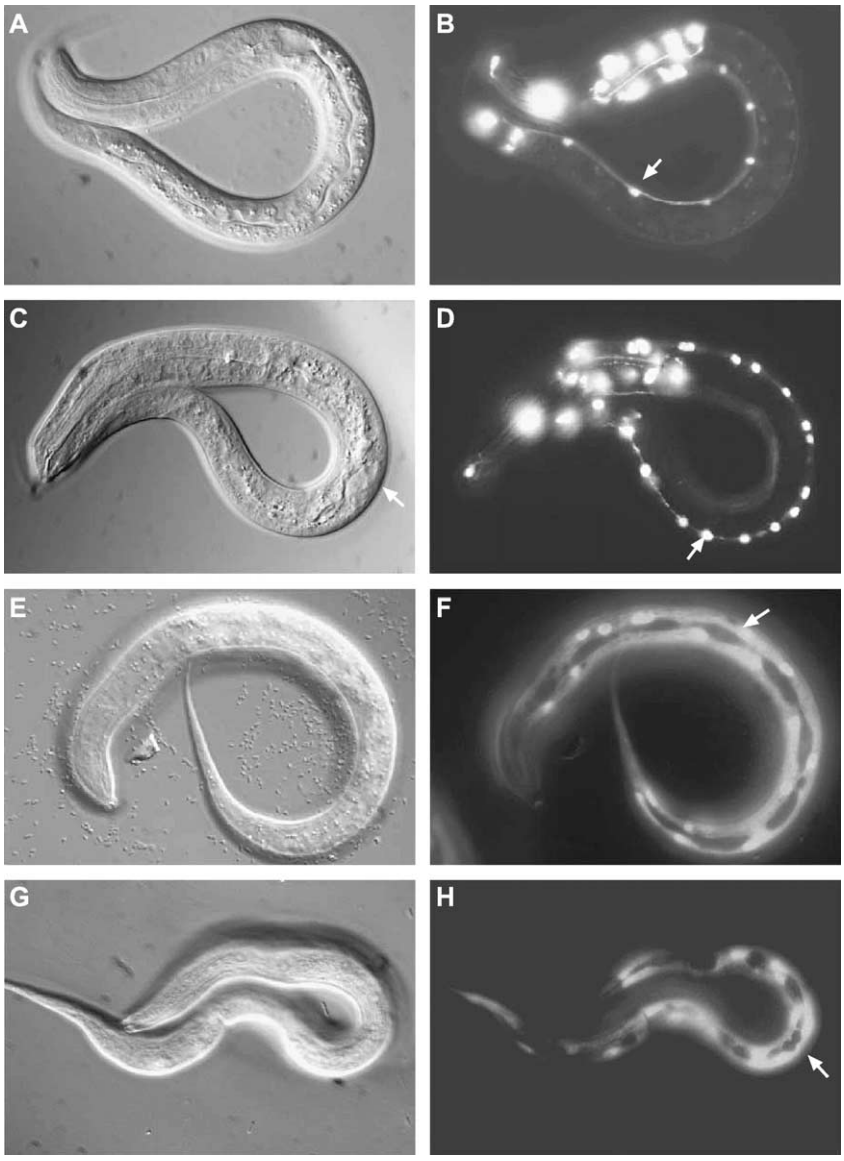

Fig. 5. The synthetic larval arrest observed in *xnp-1(tm678);lin-35*(RNAi) worms is not linked to arrested cell division. Nomarski (A, C, E, G) and fluorescent (B, D, F, H) images of *xnp-1* mutants carrying an *unc-53*::GFP (A–D) or a *nlp-29*::GFP (E–H) reporter transgene, observed as newly hatched L1 larvae (A, B, E, F), or 48 h after being laid at 25°C following *lin-35* RNAi (C, D, G, H). In the newly hatched L1 larvae, the embryonic DA motoneurones in the ventral cord are visible (B, arrow), as are the undivided seam cells (F, arrow), revealed by contrast with reporter gene expression in the hypodermis. While the size of the arrested larvae does not increase, cell divisions continue, as witnessed by the presence of the postembryonic AS motoneurones in the ventral cord (D, arrow) and the appearance of daughter seam cells (H, arrow). The gonad primordium is indicated by an arrow in C.

29::GFP (Fig. 5; data not shown). This latter finding indicates that the disappearance of *sur-5*::GFP expression in the hypodermal cells does not reflect a general cellular dysfunction or atrophy.

While transgene expression was not reduced in either *xnp-1(tm678)* or *hpl-2*(RNAi) animals, following *lin-35* RNAi in the wild-type background, *sur-5*::GFP expression in the hypodermis and intestinal cells was reduced but not abolished. These differences were quantified, using the COPAS worm sorter, confirming the qualitative observations (Fig. 7). *lin-35* has previously been reported to modulate context-dependent gene silencing (Hsieh et al., 1999). These results suggest that *xnp-1* may also play a role in this phenomenon.

Discussion

xnp-1 is required for gonadogenesis

We have shown that the *C. elegans XNP/ATR-X* homologue *xnp-1* is required for embryogenesis and somatic gonad development at 25°C and to a lesser degree at 20°C. As *xnp-1(tm678)* is a null allele, the temperature-

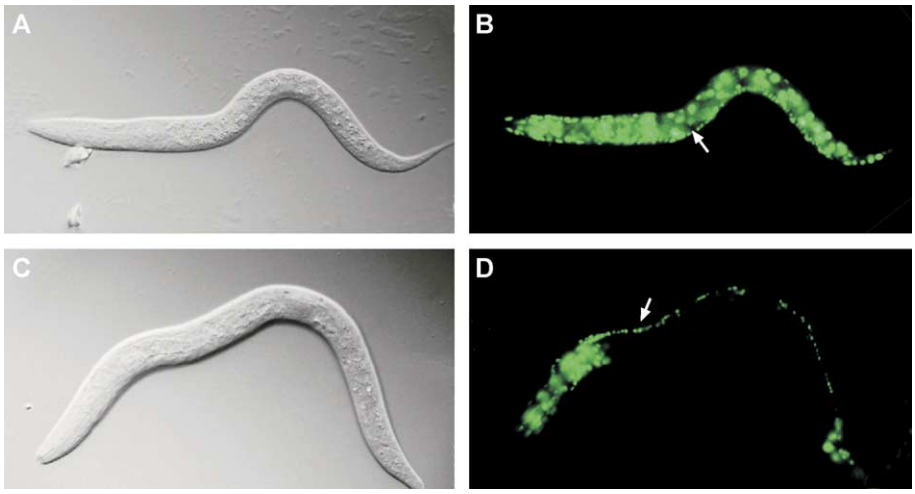

Fig. 6. Extinction of *sur-5*::GFP expression in *xnp-1;lin-35* arrested larvae. Nomarski (A, C) and fluorescent images (B, D) of *xnp-1(tm678);sur-5*::GFP transgenic L1 larvae treated with control RNAi (A, B) or *lin-35* RNAi (C, D) viewed 18 h after being laid at 25°C. *sur-5*::GFP is ubiquitously expressed in the nuclei of *xnp-1(tm678)* animals (B). Expression disappears in the hypodermis and intestine of *xnp-1*, *lin-35* larvae, while expression is maintained for example in the ventral nerve cord (arrow).

sensitive phenotype suggests that the process involving XNP-1 is temperature sensitive. Several SynMuv genes, including *lin-13* (Melendez and Greenwald, 2000), also give temperature sensitive phenotypes when mutated. Temperature is known to be a factor that can modify chromatin structure and consequently influence gene expression, as demonstrated by position effect variegation in *Drosophila* (Reuter and Spierer, 1992; Singh, 1994). The gonadogenesis defect seen in *xnp-1* mutants is similar to that obtained when the zygotic contribution of *hda-1* is removed. HDA-1 is one of the 3 *C. elegans* class I histone deacetylases, and is a component of the NuRD complex (Dufourcq et al., 2002). Removal of both zygotic and maternal contributions of *hda-1* is associated with an embryonic lethality. Surprisingly, mutation of *xnp-1* is able to partially suppress this lethality (Table 2), suggesting that the two genes could have an antagonist action in early development, but not in gonadogenesis.

xnp-1 acts redundantly with lin-35/Rb, hlp-2/HP1 and members of the NuRD complex

We found that abrogation of *xnp-1* function together with that of a subgroup of SynMuvB genes, including *lin-35*/Rb, *hpl-2*/HP1, and components of the NuRD complex *let-418*/Mi2 and *lin-53* resulted in a highly penetrant larval arrest. This synthetic larval arrest reinforces the emerging picture that the function of SynMuvB genes in *C. elegans* is not restricted to vulval cell fate determination (Boxem and van den Heuvel, 2002; Fay et al., 2002; Unhavaithaya et al., 2002). In higher organisms, the tumour suppressor gene Rb is well known to play a critical role in maintaining cells in a

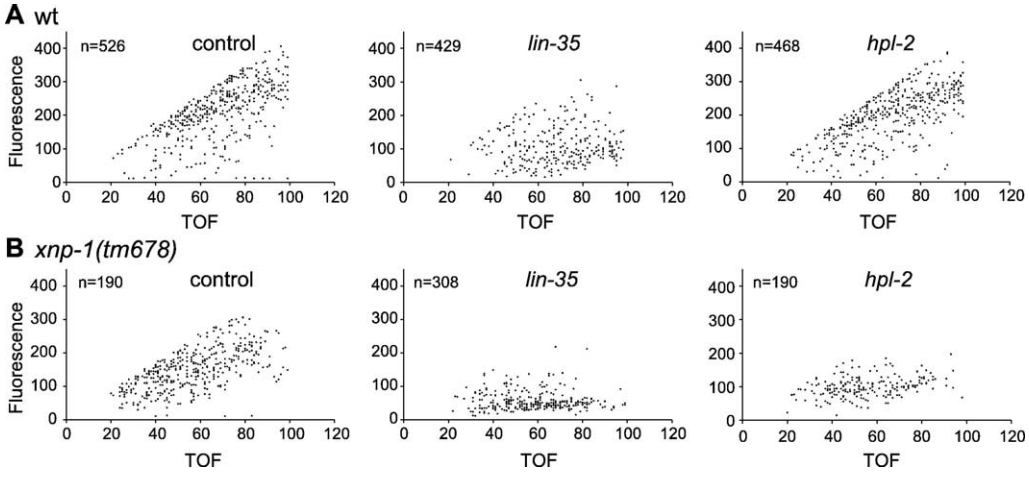

Fig. 7. Effect of inactivation of *xnp-1*, *lin-35* and *hpl-2* on *sur-5*::GFP expression. Wild type (A) or *xnp-1* mutants (B) carrying a *sur-5*::GFP reporter transgene were treated with control, *lin-35* and *hpl-2* RNAi as in Fig. 6 before quantification of GFP expression using the COPAS biosort. The fluorescence and TOF are measured in arbitrary, but constant units. The mean and SEM of the fluorescence for wild type worms are 224.0 ± 4.0, 113.3 ± 2.9 and 206.1 ± 3.7, following RNAi with control, *lin-35* and *hpl-2*, respectively, and for *xnp-1* worms 99.3 ± 2.2, 59.8 ± 3.1 and 99.3 ± 2.2.

state of growth arrest through silencing the expression of cell cycle genes. RB recruits chromatin-remodelling proteins such as histone deacetylases (Zhang et al., 2000) and HP1 (Nielsen et al., 2001) to repress the expression of E2F target genes. Several studies have shown recently that Rb also has a key role in terminal differentiation via activation of tissue-specific gene expression (reviewed in Liu et al., 2004).

We have found that inactivation of *xnp-1* greatly accentuates the silencing of a *sur-5*::GFP tandemly repeated transgenic array provoked by *lin-35*/Rb RNAi and causes a synthetic silencing phenotype when combined with *hpl-2*/HP1 RNAi. It has been proposed that certain class-B synMuv factors, including the RING finger/B-box factor TAM-1 and LIN-35/RB, modulate context-dependent silencing of specific multicopy transgenes. In addition, they may regulate endogenous genes in areas of repetitive sequence (Hsieh et al., 1999). Since loss of expression of *sur-5*, which encodes an acetoacetyl-CoA synthetase is not lethal in itself (Gu et al., 1998), we would predict that the deregulation of other, as yet uncharacterised, endogenous genes leads to the synthetic L1 larval arrest observed in *xnp-1;lin-35*(RNAi) and *xnp-1;hpl-2*(RNAi) animals.

Another SNF2 helicase LET-418 gives an L1 larval arrest phenotype when both the maternal and the zygotic contributions are removed. It is interesting to note that *xnp-1* RNAi phenocopied the removal of the *let-418* maternal contribution with regards to overall development (Table 1). But unlike *let-418* L1 arrested larvae that show ectopic expression of several germline proteins in the soma, including PGL-1 (Unhavaithaya et al., 2002), no PGL-1 ectopic expression was observed in the hypoderm or in the intestine of *xnp-1;lin-35*(RNAi) and *xnp-1;hpl-2*(RNAi) L1 arrested larvae (N.P., unpublished data) suggesting that XNP-1 and LET-418 act via different mechanisms.

One striking finding was that the arrested larvae did not increase in overall size after hatching, despite the continuation of cell divisions in all examined tissues that gave rise to the number of daughter cells normally found at the L1/L2 moult. In wild-type larvae, growth is mainly achieved by increasing cell size and endoreduplication in the hypodermis (Flemming et al., 2000). XNP-1 and Rb might therefore be required for cell growth and not be directly linked to cell cycle arrest. In support of such an idea, unlike *lin-35*/Rb and *lin-9*, several of the SynMuvB genes that give a synthetic arrest with *xnp-1*, namely *let-418*, *lin-53* and *lin-37*, do not negatively regulate G1 progression (Boxem and van den Heuvel, 2002).

Redundancy: parallel or concerted action

Synthetic phenotypes often reflect the existence of redundant pathways involving distinct intracellular signalling cassettes. Given that *xnp-1* mutants do not resemble *lin-35*/Rb mutants, our results would suggest that these two genes function in redundant pathways. A simple model would be that these factors act in parallel in distinct complexes on the regulation of different genes redundant for larval development. Indeed, recent work on the function of SynMuv genes in the specification of the vulva suggest that different chromatin remodeling complexes act redundantly in different developmental pathway (Ceol and Horvitz, 2004). XNP-1 might be a component of a complex with different functions, redundant with Rb in larval development, but essential in gonadogenesis. Alternatively, the function of the XNP-1 complex could be the same, but the process of gonadogenesis might be more sensitive to loss of *xnp-1* function than is larval development.

The partial overlap of the observed *sur-5*::GFP silencing phenotypes between *xnp-1* and *lin-35*/Rb could be explained by the concerted activity of distinct complexes on the same target genes or by additive effects due to misregulation of distinct genes. The hypothesis of XNP-1 and Rb acting in different complexes is reinforced by the recent studies suggesting that XNP/ATR-X functions in conjunction with the transcription cofactor Daxx in a novel chromatin-remodelling complex, distinct from the SWI/SNF or NURD complexes, that may remodel chromatin differently (Tang et al., 2004; Xue et al., 2003).

On the other hand, some SynMuvA and SynMuvB proteins, by definition functionally redundant, are known to participate in the same multi-protein complex (i.e., the NuRD complex), and we have found by co-immunoprecipitation that XNP/ATR-X interacts with the RB protein in human lymphoblastoid cells (C.M.R. and C.C., unpublished data). Added to the fact that HP1 has been shown to interact physically with XNP/ATR-X and with Rb, this opens the possibility that XNP-1, LIN-35/RB and HP1 function redundantly but in the same complex. Depletion of two components but not one would destabilise the complex. One model is that XNP-1 and HP1 could be recruited by LIN-35/RB and the NuRD complex, perhaps with TAM-1, to regulate the expression of specific genes during development, via chromatin remodelling. Such a complex would also affect the expression of tandemly repeated transgenic arrays. In the case of *sur-5*::GFP, it would either act directly as a positive regulator or function as a negative regulator of a repressor. While an positive role of RB on the expression of *lin-39* homeobox gene in the vulva has been described (Chen and Han, 2001), the second hypothesis is supported by the known function of the RB/HP1 complex as a repressor of transcription. As the SNF2 helicase LET-418/Mi2 is part of the NuRD complex, the recruitment of another SNF2 helicase, XNP-1, could be required for the full repression of gene expression or for the repression of a different set of genes.

XNP-1 and XNP/ATR-X

The requirement of *xnp-1* in gonadogenesis is a tantalising observation given that in humans, XNP/ATR-X mutation is associated with urogenital defects in 80% of

patients (Gibbons and Higgs, 2000). Moreover, the requirement of *xnp-1* with *lin-35*/Rb in larval development in *C. elegans* can be compared with the observation that in humans, XNP/ATR-X mutation is also associated, with short stature (66%) as well as skeletal abnormalities (91%) (Gibbons and Higgs, 2000). In contrast to humans, the developmental defect in *C. elegans* is, however, dependent upon the coordinated inactivation of SynMuvB genes and *xnp-1*.

It is also interesting to correlate the role of *xnp-1* in association with *lin-35*/Rb in context-dependent silencing of specific multicopy transgenes with the potential epigenetic regulatory function of the human XNP/ATR-X. Indeed, XNP/ATR-X is found in highly repeated regions associated with pericentromeric heterochromatin and at the nucleolar organiser region of acrocentric chromosomes that contain the ribosomal (rDNA) genes (McDowell et al., 1999). In addition, it has been shown that mutations in XNP/ATR-X patients can cause a change in the pattern of methylation of several repeated sequences including ribosomic DNA, a Y-specific sequence and subtelomeric repeats (Gibbons et al., 2000). These results and the fact that several of the genetic interaction partners of *xnp-1* are epigenetic regulators of condensed chromatin states in mammals, such as HP1, mean that it will be interesting to investigate if the human XNP/ATR-X protein is directly involved in the regulation of the expression of specific genes that remain to be discovered.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.10.014.

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