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C. elegans ISWI and NURF301 antagonize an Rb-like pathway in the determination of multiple cell fates

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The class A, B and C synthetic multivulva (synMuv) genes act redundantly to negatively regulate the expression of vulval cell fates in *Caenorhabditis elegans.* The class B and C synMuv proteins include homologs of proteins that modulate chromatin and influence transcription in other organisms similar to members of the Myb-MuvB/dREAM, NuRD and Tip60/NuA4 complexes. To determine how these chromatin-remodeling activities negatively regulate the vulval cell-fate decision, we isolated a suppressor of the synMuv phenotype and found that the suppressor gene encodes the *C. elegans* homolog of *Drosophila melanogaster* ISWI. The *C. elegans* ISW-1 protein likely acts as part of a Nucleosome Remodeling Factor (NURF) complex with NURF-1, a nematode ortholog of NURF301, to promote the synMuv phenotype. *isw-1* and *nurf-1* mutations suppress both the synMuv phenotype and the multivulva phenotype caused by overactivation of the Ras pathway. Our data suggest that a NURF-like complex promotes the expression of vulval cell fates by antagonizing the transcriptional and chromatin-remodeling activities of complexes similar to Myb-MuvB/dREAM, NuRD and Tip60/NuA4. Because the phenotypes caused by a null mutation in the tumor-suppressor and class B synMuv gene *lin-35* Rb and a gain-of-function mutation in *let-60* Ras are suppressed by reduction of *isw-1* function, NURF complex proteins might be effective targets for cancer therapy.

KEY WORDS: ISWI, NURF, Rb, Ras, *C. elegans*

INTRODUCTION

The ordered recruitment of factors required for proper gene expression is crucial for animal development. For example, sequence-specific transcription factors recruit a variety of protein complexes that remodel chromatin to regulate the transcription of target genes either by using the energy of ATP hydrolysis to move nucleosomes or by chemically modifying histones (Narlikar et al., 2002). These two mechanisms for chromatin remodeling have been characterized extensively in vitro (Roth et al., 2001; Smith and Peterson, 2005), but efforts to understand how each functions in the development of metazoa have just begun.

Studies of vulval development in the nematode *Caenorhabditis elegans* could help establish the roles of chromatin remodeling during development. The vulva of the *C. elegans* hermaphrodite is formed by the 22 descendants of three ectodermal blast cells (P5.p, P6.p and P7.p) located along the ventral surface of the animal (Sulston and Horvitz, 1977). P(5-7).p, three of a set of six equipotent cells P(3-8).p called the vulval equivalence group, are induced to generate vulval cells by an epidermal growth factor (EGF)-like signal from the gonad (Sulston and White, 1980; Hill and Sternberg, 1992). The inductive signal is received and transduced by a conserved receptor tyrosine kinase (RTK)/Ras pathway (Kornfeld, 1997). Unlike the other cells of the vulval equivalence group (P3.p, P4.p and P8.p), which divide once and fuse with the nearby hypodermal syncytium (hyp7), P(5-7).p divide three times to generate the cells that form the adult vulva (Sulston and Horvitz, 1977). Mutations that reduce or eliminate the function of the *let-23* RTK/*let-60* Ras pathway can result in a vulvaless (Vul) animal in which no cells of the vulval equivalence group express vulval fates; by contrast, mutations that increase the function of this pathway can cause the ectopic adoption of vulval cell fates by P3.p, P4.p and P8.p, and result in a multivulva (Muv) animal (Beitel et al., 1990; Han et al., 1990; Katz et al., 1996).

Loss-of-function mutations in the synthetic multivulva (synMuv) genes also can cause a Muv phenotype (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989). These genes have been grouped into three classes: A, B and C (Ferguson and Horvitz, 1989; Ceol and Horvitz, 2004). Loss-of-function mutations within any one class do not cause a Muv phenotype, whereas mutations in any two genes within two different classes cause a Muv phenotype (Ferguson and Horvitz, 1989; Ceol and Horvitz, 2004). The class A synMuv genes encode novel, nuclear components (Clark et al., 1994; Huang et al., 1994; Davison et al., 2005). Many class B synMuv genes encode homologs of transcriptional repressors and factors that remodel chromatin, including LIN-35 Rb (Lu and Horvitz, 1998), the EFL-1/DPL-1 E2F heterodimeric transcription factor (Ceol and Horvitz, 2001), the HDA-1 HDAC1, LET-418 Mi2, LIN-53 RbAp48 NuRD complex (Lu and Horvitz, 1998; Tong et al., 1998; Xue et al., 1998; von Zelewsky et al., 2000; Unhavaithaya et al., 2002) and HPL-2 Heterochromatin Protein 1 (HP1) (Couteau et al., 2002). The *Drosophila melanogaster* homologs of some class B synMuv proteins form a complex, identified by two different groups and called Myb-MuvB or dREAM, that is likely to repress the transcription of genes through chromatin remodeling (Korenjak et al., 2004; Lewis et al., 2004). Class C synMuv genes encode homologs of a putative Tip60/NuA4 histone acetyltransferase complex (Ceol and Horvitz, 2004). Because of these homologies, the synMuv genes, which negatively regulate the vulval cell fate, probably act by repressing the transcription of genes that promote the expression of vulval cell fates.

In this study, we describe the identification of a *C. elegans* ortholog of *Drosophila ISWI*, called *isw-1*, as a suppressor of the synMuv phenotype. ISWI is an ATP-dependent chromatin-remodeling enzyme identified by homology to *S. cerevisiae*

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Snf2/Swi2 (Elfring et al., 1994). We show that ISW-1 probably acts as a component of a Nucleosome Remodeling Factor (NURF)-like complex with the *Drosophila* NURF301 ortholog NURF-1 to antagonize the synMuv genes. Our observations reveal the antagonistic functions of a NURF-like chromatin remodeling complex and complexes similar to Myb-MuvB/dREAM, NuRD and TIP60/NuA4 in the determination of multiple cell fates, including the antagonistic regulation of at least one putative target of synMuv transcriptional repression.

MATERIALS AND METHODS

Strains and genetics

C. elegans strains were cultured as described previously and maintained at 20°C unless otherwise noted (Brenner, 1974). N2 (Bristol) was the wild-type strain. The following mutations and integrants were used:

LGI: *dpy-5(e61)*, *lin-35(n745)*, *lin-53(n833, n3368)* (*n3368*, this study), *ccIs4251* (Hsieh et al., 1999).

LGII: *unc-4(e120)*, *nurf-1(n4293*, *n4295)* (this study), *dpl-1(n3316)* (Ceol and Horvitz, 2001), *lin-8(n111, n2731)* (Thomas et al., 2003), *lin-56(n2728)* (Thomas et al., 2003), *lin-38(n751)*, *trr-1(n3712)* (Ceol and Horvitz, 2004), *lin-31(n301)*, *let-23(sa62)* (Katz et al., 1996), *rrf-3(pk1426)* (Simmer et al., 2002).

LGIII: *dpy-17(e164)*, *isw-1(n3294*, *n3297*, *n4066)* (this study), *lin-9(n112*, *n942)* (Beitel et al., 2000), *lin-36(n766)* (Thomas and Horvitz, 1999), *lin-37(n758*, *n2234)* (Ferguson and Horvitz, 1989; Thomas et al., 2003), *lin-52(n3718)* (Thomas et al., 2003), *lin-13(n387)* (Melendez and Greenwald, 2000), *hpl-2(tm1489)*, *flt-1(tm235)*.

LGIV: *unc-30(e191)*, *pyp-1(n4599)* (this study), *let-60(n1046)* (Beitel et al., 1990; Han et al., 1990), *lin-1(n304*, *e1275)*.

LGV: *unc-46(e177)*, *let-418(n3536, n3719)*, *mep-1(q660)*, *lin-54(n3423)*, *hda-1(e1795)* (Dufourcq et al., 2002), *tam-1(cc567)* (Hsieh et al., 1999), *lin-45(ku112)* (Sundaram and Han, 1995), *him-5(e1490)*.

LGX: *lin-15A(n767, n433)*, *lin-15B(n744)*, *lin-15AB(n765, e1763)*, *lin-2(n768)*.

The following reciprocal translocations containing GFP-expressing transgenes integrated at or near the translocation breakpoints were used: *hT2 [qIs48]* LGI; LGIII and *nT1 [qIs51]* LGIV; LGV. *mIn1 [mIs14 dpy-10(e128)]* is a balancer chromosome that expresses GFP. The following mutations were provided by C. Ceol, F. Stegmeier and M. Harrison: *let-418(n3536*, *n3719)*, *lin-54(n3423)*, *mep-1(n3703)*. *flt-1(tm235)* and *hpl-2(tm1489)* were provided by S. Mitani. Those mutant alleles for which no citation is given are described by Riddle (Riddle, 1997).

RNAi analyses

RNAi by injection was performed as described by Ceol and Horvitz (Ceol and Horvitz, 2004), except single-stranded RNA molecules were annealed by incubation at 85°C for 15 minutes, then at 37°C for 30 minutes and slowly cooled to room temperature for 1 hour. RNAi of *F37A4.6*, the gene predicted to be within an intron of *isw-1*, did not suppress the synMuv phenotype of *lin-53(n833); lin-15A(n767)* mutants (data not shown). The following constructs were used to make dsRNA: for *isw-1*, yk593a10 and yk617c10; for *nurf-1*, yk273g2, yk1151c6, pEA30 (a cDNA encoding the 3′ end of *nurf-1b*, *c*, *d* and *e*) and pEA147 (a RT-PCR product corresponding to only *nurf-1a*); for *pyp-1*, yk169c6; for *rba-1*, yk117c9; for *H20J04.2*, yk230c2 and yk323f2; for *T26A5.8*, yk471d3; and for*Y53F4B.3*, yk393b2 and yk1412b12. Yuji Kohara kindly provided all yk clones.

Determination of gene structures

For *isw-1*, the sequences of two independent cDNAs, yk593a10 and yk617c10, were determined. 5′ rapid amplification of cDNA ends (5′ RACE, Invitrogen) was used to determine the 5′ end of *isw-1*, and an SL1 splice-leader sequence was detected. For *nurf-1*, the sequences of 15 independent cDNAs were determined: yk62e9 (pEA30), yk98g1, yk172b9, yk374b9, yk381c2, yk480b9, yk565d9, yk752a4, yk765d8, yk879b11, yk1030g7, yk1151c6, yk1288b1, yk1456g11, yk1691d5. Gene-specific primers were used to amplify reverse-transcribed products by PCR to confirm several *nurf-1* transcripts, and a Stratagene *C. elegans* cDNA library also was used for PCR analyses of *nurf-1* gene structures. The 5′ ends of *nurf-1b* and *nurf-1c* were identified by SL1 RT-PCR, and the 5′ ends of *nurf-1a*, *d* and *e* are from GeneFinder (Liang et al., 2001) predictions.

Isolation of deletion alleles

Genomic DNA pools from EMS-mutagenized animals were screened for deletions using PCR as described by Ceol and Horvitz (Ceol and Horvitz, 2001). Deletion mutant animals were isolated from frozen stocks and backcrossed to the wild type at least twice. *isw-1(n4066)* removes nucleotides 20629 to 21932 of cosmid F37A4. *nurf-1(n4293)* removes 3058 to 3782, and *nurf-1(n4295)* removes 18656 to 19733 of cosmid F26H11. *pyp-1(n4599)* removes nucleotides 26777 to 28936 of cosmid C47E12. *lin-53(n3368)* removes nucleotides 38104 to 38857 of cosmid K07A1.

Scoring of vulval cell fates

For *trr-1(n3712)* and *trr-1(n3712); lin-15B(n744)*, vulval induction was scored during the L4 larval stage using Nomarski optics. If more than three out of the six Pn.p cells were induced, the animals were counted as Muv.

Antibody staining

We cloned a full-length *isw-1* cDNA into a vector containing the coding sequence for the maltose-binding protein (MBP). Antisera recognizing ISW-1 were generated by injecting MBP:ISW-1 into two rabbits (Covance). Anti-ISW-1 antibodies were affinity purified using GST (glutathione S-transferase)-tagged ISW-1 as described by Koelle and Horvitz (Koelle and Horvitz, 1996). Embryos, larvae and adults were fixed as described by Finney and Ruvkun (Finney and Ruvkun, 1990). Affinity-purified antibodies were used at a 1:10 dilution for whole-mount staining and at 1:1000 for western blots. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:3000 for western blots, and Alexfluor 488 (Invitrogen) was used at a 1:200 dilution for detection by whole-mount immunocytochemistry.

Determination of mutant sequences

We used PCR-amplified regions of genomic DNA to determine mutant sequences. For both *isw-1* alleles, all exons and splice junction sequences were determined. All mutations were confirmed using independently derived PCR products. Sequences were determined using an ABI Prism 3100 Genetic Analyzer.

Germline transformation experiments

Germline transformation experiments were performed as described by Mello et al. (Mello et al., 1991). For rescue of the *lin-53(n833); isw-1(n3294); lin-15A(n767)* synMuv suppression phenotype, we injected cosmid C28G2 (30 ng/μl). 100 μg/μl of 1 kb DNA ladder (Invitrogen) was used to increase the complexity of the extrachromosomal arrays, and pTG96 (*sur-5::gfp*) (Yochem et al., 1998) was used as the co-injection marker at 20 ng/μl.

Suppression of non-vulval defects caused by class B synMuv mutations

Using the same exposure time, GFP expression (Tam phenotype) was quantified for each micrograph within the linear range for signal detection using the Profiler function of the OpenLab software package (Improvision). PGL-1 staining and RNAi sensitivity were scored as described by Wang et al. (Wang et al., 2005). The L1 larval arrest phenotypes of *let-418(n3536)* and *mep-1(n3701)* were scored at 25°C.

RESULTS

Loss of function of the *C. elegans* homolog of *Drosophila* ISWI causes suppression of the *lin-53; lin-15A* synMuv phenotype

We screened for suppressors of the *lin-53(n833); lin-15A(n767)* synMuv phenotype and identified two mutations, *n3294* and *n3297*, that failed to complement for suppression of the synMuv phenotype (Fig. 1A; Table 1). We mapped the synMuv suppressor *n3294* between *sma-3* and *unc-36* on LGIII and obtained transformation rescue of the synMuv suppression phenotype with cosmid C28G4, which contains 16 predicted genes. RNA interference (RNAi) of the

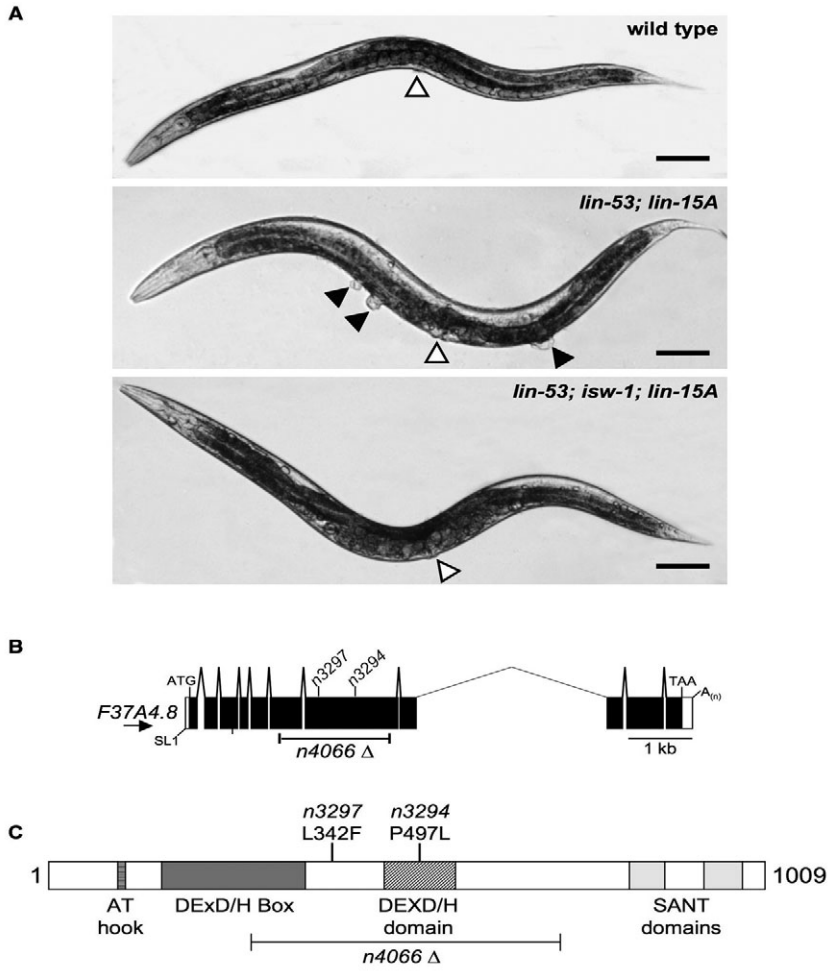


Fig. 1. Loss-of-function mutations in *isw-1* suppress the synMuv phenotype of *lin-53; lin-15A* mutants. (**A**) Bright-field micrographs of *C. elegans* 24 hours after the fourth larval stage. Vulvae and psuedovulvae are marked with white triangles and black triangles, respectively. (Top) The wild-type strain has no pseudovulvae. (Middle) The synMuv mutant *lin-53(n833); lin-15A(n767)* has three pseudovulvae. (Bottom) The *lin-53(n833); isw-1(n3294); lin-15A(n767)* triple mutant has no pseudovulvae. Bacteria, embryos or bubbles were removed from the images. Scale bar: 100 μm. (**B**) The genomic structure of *isw-1*. Exons are indicated by black boxes; 5′ and 3′ untranslated regions are indicated by white boxes. Predicted translation initiation and termination codons and the polyadenylation site are shown. The locations of the missense mutations and deletion allele are indicated. (**C**) A representation of the domain structure of the ISW-1 protein. ISW-1 is similar to *Drosophila* ISWI and other family members, especially in the named domains (see text). The amino acid substitutions of the two missense alleles and the locations of these mutations and the deletion allele are indicated. An alignment of ISW-1 with *S. cerevisiae* SWI2/SNF2 and *Drosophila* ISWI is in Fig. S1.

gene *F37A4.8* (using either of two cDNA clones; see Materials and methods) caused suppression of the *lin-53; lin-15A* synMuv phenotype. Additionally, expression of an *F37A4.8* cDNA driven by a *dpy-7* promoter (Gilleard et al., 1997) rescued the synMuv suppression phenotype of *n3294* animals (Table 1). We determined that *n3294* and *n3297* harbor two distinct missense mutations in *F37A4.8* (Fig. 1B; see Fig. S1 in the supplementary material).

Using two independent cDNA clones and one 5′ RACE product (see Materials and methods), we determined the sequence of the full-length *F37A4.8* transcript, which included the SL1 *trans*-splice leader sequence found in many *C. elegans* transcripts (Blumenthal, 1995) (Fig. 1B). *F37A4.8* encodes the *C. elegans* homolog of *Drosophila* ISWI, a regulator of transcription and chromatin (Elfring et al., 1994; Deuring et al., 2000). We named *F37A4.8 isw-1* (*isw*, ISWI-like).

ISW-1 is 60% identical to *Drosophila* ISWI (Elfring et al., 1994) and 69% identical to human SNF2H (Okabe et al., 1992). ISW-1 contains an AT-hook (Reeves and Nissen, 1990) and two SANT (Aasland et al., 1996) domains; each of these domains can bind DNA. Additionally, ISW-1 contains a domain similar to many helicases (the DEXD/H box) and an ATPase domain. Each are required for chromatin-remodeling activity (Corona et al., 1999). *isw-1(n3294)* is predicted to cause a proline-to-leucine substitution within the DEXD/H domain, implicating this domain in *isw-1* function. *isw-1(n3297)* is predicted to cause a leucine-to-phenylalanine substitution within a non-conserved region (Fig. 1C; see Fig. S1 in the supplementary material).

isw-1(n3294) and *isw-1(n3297)* each conferred a fully penetrant recessive synMuv suppression and incompletely penetrant sterile phenotype (Table 1; see Table S1 in the supplementary material). By contrast, RNAi of *isw-1* caused a fully penetrant synMuv suppression and sterile phenotype (Table 1; see Table S1 in the supplementary material). We isolated a deletion allele of *isw-1*, *n4066*, which removes part of the ATPase domain and most of the DEXD/H domain, causing a presumptive null phenotype. The *isw-1(n4066)* deletion allele caused a fully penetrant recessive sterile phenotype (see Table S1 in the supplementary material) but failed to cause strong suppression of the synMuv phenotype (Table 1). Because *isw-1(n4066)* homozygotes are sterile, the animals we studied were descended from *isw-1(n4066)* heterozygotes. Homozygous missense mutants descended from heterozygous mothers also did not have a strong synMuv suppression phenotype (Table 1), so the lack of synMuv suppression observed in *isw-1* homozygotes derived from heterozygous mothers was probably caused by maternally inherited wild-type *isw-1* gene product. *isw-1(n4066)* did perturb the synMuv suppressor function of *isw-1*, because this mutation failed to complement the synMuv suppression phenotype caused by each of the missense alleles (Table 1). *isw-1(n3294)* and *isw-1(n3297)* cause a decrease of *isw-1* function, because each resulted in recessive suppression of the synMuv phenotype and failed to complement the phenotype conferred by a deletion of *isw-1*. Additionally, RNAi of *isw-1* caused a synMuv suppression and sterile phenotype, so the two *isw-1* missense alleles probably cause a partial loss of *isw-1* gene function.

Table 1. Loss of *isw-1* function suppresses the synMuv phenotype of *lin-53(n833); lin-15A(n767)* mutants

isw-1 genotype in a *lin-53; lin-15A* mutant	% Muv (*n*)
+/+	100 (319)
n3294 M⁻*	0 (230)
n3294 M+†	61 (76)
n3294/+ M+ ‡	100 (40)
n3294/+ M⁻ §	63 (130)
n3297 M⁻	0 (121)
n3297 M+†	64 (56)
n3297/+ M+‡	100 (53)
n3297/+ M–§	81 (111)
n3294/n3297	0 (103)
n4066 ¶ M+	95 (212)
n4066/+ M+	100 (128)
n3294/n4066	0 (58)
n3297/n4066	0 (38)
isw-1 RNAi	0 (353)
n3294; dpy-7::isw-1(+)	96 (177)

*M+ or M– denotes the presence (+) or absence (–) of maternal *isw-1* gene product. M+ animals were descended from *isw-1* heterozygous mutant hermaphrodites. M– animals were descended from *isw-1* homozygous mutant hermaphrodites.
†These animals were the non-GFP-positive offspring of *lin-53(n833); isw-1/qC1 [nIs188]; lin-15A(n767)* hermaphrodites.
‡These animals were the non-Dpy non-GFP-positive offspring of *dpy-5(e61) lin-53(n833); lin-15A(n767)* hermaphrodites and *lin-53(n833)/ hT2 [qIs48]; isw-1/hT2 [qIs48]; lin-15A(n767)* males.
§These animals were the non-Dpy offspring of *dpy-5(e61) lin-53(n833); isw-1; lin-15A(n767)* hermaphrodites and *lin-53(n833); him-5(e1490); lin-15A(n767)* males.
¶ *isw-1(n4066)* caused recessive sterility, so homozygous offspring from heterozygous hermaphrodites were scored. *isw-1(n4066)* was *cis*-marked with *dpy-17(e164)*.
For the dominance, complementation and maternal effect tests with the missense alleles, a *dpy-5(e61) cis*-marked *lin-53(n833)* strain was used.

ISW-1 is nuclear, ubiquitously expressed and associated with chromatin

We generated specific antibodies that recognized a protein of the predicted ISW-1 size (~115 kDa) by western blot analysis of wild-type but not *isw-1(n4066)* adults (see Fig. S2A in the supplementary material). ISW-1 was present in the nuclei of most, if not all, cells during every stage of *C. elegans* development (see Fig. S2B-D in the supplementary material). ISW-1 was associated with chromatin, as indicated by colocalization of anti-ISW-1 immunoreactivity with DAPI-stained condensed chromosomes (see Fig. S2E in the supplementary material).

Decreased function of the *C. elegans* homolog of *Drosophila nurf301* suppresses the synMuv phenotype

In *Drosophila*, ISWI acts as the ATPase subunit of several ATP-dependent chromatin-remodeling complexes, including ACF (Ito et al., 1997), CHRAC (Varga-Weisz et al., 1997) and NURF (Tsukiyama and Wu, 1995). We determined whether any of the *C. elegans* genes encoding presumptive components of homologous complexes act similarly to *isw-1* to promote the synMuv phenotype.

Using BLAST (Altschul et al., 1990) and SMART (Sonnhammer et al., 1997) searches, we identified *C. elegans* orthologs of the ACF, CHRAC and NURF complex members. The ACF and CHRAC complexes share one component: ACF1. Deletion of one of the two genes encoding a *C. elegans* ACF1 ortholog (*flt-1*), RNAi of the other ortholog (*H20J04.2*), or both deletion and RNAi together failed to suppress the synMuv phenotype. Furthermore, RNAi of each of the remaining genes encoding CHRAC complex orthologs failed to suppress the synMuv phenotype (Table 2). In these RNAi experiments in which a failure to suppress the synMuv phenotype was observed, it remains possible that the gene plays a role in the antagonism of the synMuv genes, but this role was not seen because the gene was not inactivated sufficiently.

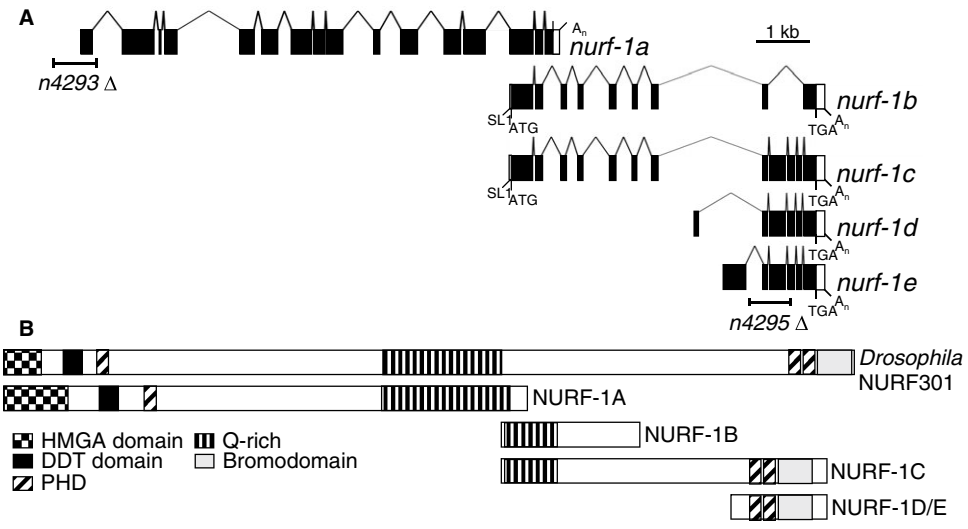
Only RNAi of a *Drosophila* NURF301 ortholog, which we named *nurf-1* (NURF301-like), suppressed the synMuv phenotype (Table 2). The *Drosophila* NURF complex consists of ISWI, NURF301, NURF38 and NURF55 (Tsukiyama and Wu, 1995). RNAi of the sole *C. elegans* NURF38 ortholog, *pyp-1*, or of one of the two NURF55 orthologs, *rba-1*, caused embryonic lethality (data not shown), and thus precluded the scoring of the synMuv suppression phenotype. The other NURF55 ortholog is a class B synMuv gene, *lin-53* (Lu and Horvitz, 1998), and was not tested for synMuv suppression for this reason. Additionally, a deletion allele of *pyp-1* caused larval lethality before the third larval stage (data not shown), which precluded scoring of the synMuv suppression phenotype.

Using the sequences determined from 15 independent cDNA clones (see Materials and methods), RT-PCR products and 5′ RACE products, we identified five distinct transcripts generated from the *nurf-1* locus (Fig. 2A). Each transcript is predicted to encode a protein with domains similar to some of the domains of *Drosophila* NURF301. However, none of the identified transcripts is predicted to encode a protein with all of the domains contained in full-length NURF301 (Fig. 2B).

Table 2. Reduction of NURF-like complex genes but not ACF-like or CHRAC-like genes suppresses the synMuv phenotype

Genotype in addition to *lin-15AB(n765)*	*Drosophila* homolog(s)	*Drosophila* complex(es) in which the homolog is found	% Muv (*n*)
Wild type	–	–	100 (546)
isw-1(n3294)	ISWI	ACF, CHRAC, NURF	0 (254)
nurf-1(RNAi)	NURF301	NURF	1 (284)
pyp-1(RNAi)	NURF38	NURF	NA*
pyp-1(n4599)† M+‡	NURF38	NURF	NA*
rba-1(RNAi)	NURF55	NURF	NA*
H20J04.2(RNAi)	ACF1	ACF, CHRAC	99 (150)
flt-1(tm235)	ACF1	ACF, CHRAC	100 (158)
flt-1(tm235); H20J04.2(RNAi)	ACF1	ACF, CHRAC	100 (184)
T26A5.8(RNAi)	CHRAC-14	CHRAC	100 (122)
Y53F4B.3(RNAi)	CHRAC-16	CHRAC	100 (210)
Y53F4B.3(RNAi); T26A5.8(RNAi)	CHRAC-14 and CHRAC-16	CHRAC	100 (140)

*NA, not applicable because RNAi caused lethality prior to vulval development.
†*n4599* caused recessive larval lethality prior to vulval development.
‡M+ denotes that the maternally provided product might be present in these homozygous offspring.



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Genotype in a *lin-15AB(n765)* mutant background	Percent Muv (n)
nurf-1(+)	100 (546)
nurf-1a RNAi (pEA147)	0 (91)
nurf-1b,c,d,e RNAi (pEA30)	100 (111)
nurf-1a(n4293) M+	4 (230)
nurf-1b,c,d,e(n4295)	100 (204)

Fig. 2. The *nurf-1* locus is predicted to encode multiple proteins. Each protein shares domains with *Drosophila* NURF301. Only the isoform containing the HMGA domain is required for antagonism of the synMuv genes. (**A**) Genomic structures of the *nurf-1* isoforms. Exons are indicated by black boxes; 5′ and 3′ untranslated regions are indicated by white boxes. The predicted translation initiation and termination codons and the polyadenylation sites are indicated. The locations of the two deletion alleles are shown. (**B**) A representation of the NURF-1 protein and domain structure. (Top) The *Drosophila* NURF301 protein. (Bottom) The predicted protein products of the *nurf-1* gene. An alignment of the predicted functional domains of NURF-1 with those of *Drosophila* NURF301 is presented in Fig. S3. (**C**) Reduction of *nurf-1a* function suppresses the synMuv phenotype of *lin-15AB* mutants. RNAi of the *nurf-1a* isoform (pEA147) but not of the *nurf-1b, nurf-1c, nurf-1d, nurf-1e* isoforms (pEA30) caused suppression of the *lin-15AB* synMuv phenotype. In addition, deletion of the *nurf-1a* isoform (*n4293*) but not of the *nurf-1b, nurf-1c, nurf-1d, nurf-1e* isoforms (*n4295*) caused suppression of the *lin-15AB* synMuv phenotype. M+ denotes progeny of heterozygous mutant hermaphrodites, such progeny might retain maternally inherited *nurf-1a* gene products.

nurf-1a encodes a protein most similar to the N terminus of NURF301 and contains domains implicated in binding DNA, including an HMGY/I domain (Reeves and Beckerbauer, 2001), a DDT domain (Doerks et al., 2001) and a PHD finger (Schindler et al., 1993; Aasland et al., 1995). *nurf-1b* and *nurf-1c* share two exons with *nurf-1a* and encode proteins with similarity to the C-terminus of NURF301. Unlike *nurf-1b*, which encodes a protein with only a Q-rich domain, *nurf-1c* encodes a protein with two PHD fingers and a bromodomain. The *nurf-1d* and *nurf-1e* transcripts are initiated at different sites but encode the same predicted protein, which shares a C terminus with NURF-1C. RNAi of *nurf-1a*, but not of the other *nurf-1* transcripts, suppressed the synMuv phenotype of *lin-15AB(n765)* mutants and caused sterility (Fig. 2C and data not shown).

To confirm that reduction of the *nurf-1a* transcript was responsible for the suppression of the synMuv phenotype, we isolated two deletion alleles of the *nurf-1* locus, *n4293* and *n4295*. *nurf-1(n4293)* is deleted for part of *nurf-1a* but not for the other *nurf-1* variants. *nurf-1(n4295)* removes part of every *nurf-1* variant except *nurf-1a*. *nurf-1(n4293)* but not *nurf-1(n4295)* suppressed the synMuv phenotype of *lin-15AB(n765)* mutants and caused sterility, both features of the *isw-1* mutant phenotype (Fig. 2C and data not shown). Therefore, *nurf-1a* probably acts with *isw-1* to promote the synMuv phenotype.

The *C. elegans* NURF-like genes *isw-1* and *nurf-1* promote the synMuv phenotypes of all synMuv mutant combinations

The *C. elegans* NURF-like genes might promote the ectopic vulval fates of only specific synMuv mutant combinations, e.g. the *lin-53; lin-15A* double mutant. To address this issue, we used RNAi to reduce the function of *isw-1* or *nurf-1* in a variety of synMuv double mutants. Inactivation of *isw-1* or *nurf-1* suppressed not only the synMuv phenotype of *lin-53(n833); lin-15A(n767)* animals but also the synMuv phenotype of the null double mutant combination *lin-53(n3368); lin-15A(n767)* (Table 3). Additionally, RNAi of *isw-1* or *nurf-1* suppressed other class AB, BC and AC synMuv double mutant combinations (Table 3). Reduction of *isw-1* function suppressed the synMuv phenotype of *lin-53(n833)* in combination with putative null mutations in each of the class A synMuv genes. Additionally, reduction of *isw-1* function suppressed the synMuv phenotype of *lin-15A(n767)* in combination with putative null alleles of all identified class B synMuv genes (Table 3; see Table S2 in the supplementary material).

isw-1 and *nurf-1* promote the multivulva phenotype caused by activation of the Ras pathway

A functional Ras pathway is required for expression of the synMuv phenotype (Ferguson et al., 1987; Beitel et al., 1990; Han and Sternberg, 1990). Therefore, reduction of *isw-1* function might suppress the synMuv phenotype by reducing the activity of the Ras pathway. If so, *isw-1* might act by promoting the activity of the Ras pathway. However unlike many mutants defective in the Ras pathway, *isw-1* mutants did not have abnormalities in the expression of vulval cell fates.

We tested for a subtle role of *isw-1* in the specification of vulval cells by asking if a weak Vul phenotype conferred by a decrease in Ras pathway activity could be enhanced by an *isw-1* mutation. A *lin-2* partial loss-of-function mutation, *e1453*, causes an incompletely penetrant Vul phenotype (Ferguson and Horvitz, 1985) and a weak

Table 3. Reduction of the *C. elegans* NURF-like genes complex suppresses the synMuv phenotype of multiple synMuv mutant combinations

Genotype	% Muv after control RNAi (*n*)	% Muv after *isw-1* RNAi (*n*)	% Muv after *nurf-1a* RNAi (*n*)
*lin-53(n833)†; lin-15A(n767)**	100 (319)	0 (353)	0 (128)
lin-53(n3368); lin-15A(n767)**	100 (95)	2 (155)	0 (86)
*lin-15AB(e1763)**	100 (300)	55 (347)	40 (72)
lin-35(n745); lin-15A(n767)**	100 (143)	14 (70)	8 (62)
lin-37(n758); lin-15A(n433)†*	100 (625)	52 (431)	0 (91)
lin-37(n758); lin-15A(n767)**	100 (207)	5 (99)	21 (39)
*lin-53(n833)†; lin-8(n2731)**	100 (217)	1 (109)	0 (65)
*lin-53(n833)†; lin-56(n2728)**	100 (234)	4 (118)	0 (66)
*trr-1(n3712)M+; lin-15A(n767)**	56 (117)	0 (77)	7 (28)
*trr-1(n3712)M+; lin-15B(n744)**	49 (59)	3 (75)	23 (22)
lin-8(n111)†; lin-9(n112)†	100 (243)	1 (181)	6 (51)

lin-8, *lin-15A* and *lin-56* are class A synMuv genes. *lin-9*, *lin-15B*, *lin-35*, *lin-37* and *lin-53* are class B synMuv genes. *trr-1* is a class C synMuv gene.
*This allele is a likely complete loss-of-function mutation.
†This allele is a partial loss-of-function mutation.
M+This allele is a strong loss-of-function mutation, and the mutant phenotype is maternally rescued.
Data concerning the suppression by *isw-1* RNAi of additional synMuv double mutants are found in Table S2.

mutation in *lin-45* Raf, *ku112,* does not cause a vulval cell-fate defect (Sundaram and Han, 1995). *lin-45(ku112)* has been used to identify mutations that as single mutants do not cause a vulval-fate defect but in combination with *lin-45(ku112)* cause a synthetic vulvaless (synVul) phenotype, implicating the genes defined by such mutations in the generation of vulval cell fates (Rocheleau et al., 2002). We scored vulval induction in *isw-1; lin-2* and *isw-1; lin-45* double mutants and observed that *isw-1* did not enhance the Vul phenotype caused by *lin-2(e1453)* (42% versus 38%) and did not cause a synVul phenotype in combination with *lin-45(ku112)* (data not shown). These data suggest that if *isw-1* promotes the activity of the Ras pathway, it might act redundantly with other factors.

Gain-of-function mutations in *let-23* RTK or *let-60* Ras cause a Muv phenotype (Beitel et al., 1990; Han et al., 1990; Katz et al., 1996). Reduction of *isw-1* and *nurf-1* function partially suppressed the Muv phenotype caused by increased *let-23* and *let-60* gain-of-function mutations (Table 4). The Ras pathway terminates with the transcription factors LIN-1 ETS and LIN-31 HNF/Forkhead. *lin-1* has a primary role in inhibiting vulval cell fates, such that *lin-1* null mutants have a Muv phenotype (Beitel et al., 1995; Tiensuu et al., 2005). LIN-31 when bound to LIN-1 inhibits vulval cell fates, but after phosphorylation by MPK-1 MAPK, LIN-31 promotes vulval cell fates (Tan et al., 1998; Miller et al., 2000). *lin-31* mutants can have either a Muv or Vul phenotype, because the vulval cells are unregulated and stochastically adopt a vulval or non-vulval cell fate (Miller et al., 2000). Reduction of *isw-1* and *nurf-1* function suppressed the Muv phenotype caused by a partial loss-of-function *lin-1* mutation and a null *lin-31* mutation but failed to suppress the null *lin-1* mutant phenotype (Table 4). The failure to enhance a sensitized abnormal vulval phenotype and to suppress completely the Muv phenotype caused by an increase in Ras pathway activity suggests either that a greater inhibition of the functions of *isw-1* and *nurf-1* is required to observe complete effects or that other factors act redundantly with the NURF-like genes to promote Ras pathway activity.

Table 4. Reduction of *isw-1* or *nurf-1* function suppresses the Muv phenotype of some Ras pathway mutants

Genotype	% Muv after control RNAi (*n*)	% Muv after *isw-1* RNAi (*n*)	% Muv after *nurf-1a* RNAi (*n*)
let-23(sa62gf)	91 (57)	44 (88)	66 (27)
let-60(n1046gf)	96 (228)	19 (148)	9 (159)
lin-1(e1275)†	87 (100)	22 (107)	2 (103)
*lin-1(n304)**	100 (134)	100 (53)	100 (34)
*lin-31(n301)**	60 (312)	9 (96)	18 (246)

*This allele is a likely complete loss-of-function mutation.
†This allele is a partial loss-of-function mutation.

Reduction of *isw-1* and *nurf-1* function suppress non-vulval abnormalities of class B synMuv mutants

Several class B synMuv genes control aspects of a germline-versus-soma cell-fate decisions. Specifically, the somatic cells of some class B synMuv mutants adopt a more germline-like fate: by the somatic expression of germline genes, such as PGL-1 (Kawasaki et al., 1998); by a germline-like appearance of somatic cells in *mep-1* and *let-418* arrested larvae (Unhavaithaya et al., 2002); by the tandem-array-modifier (Tam) phenotype of increased somatic silencing of expression from repetitive transgenes by a process similar to that which occurs in the germline (Kelly et al., 1997; Kelly and Fire, 1998; Hsieh et al., 1999); and by enhanced RNAi sensitivity, perhaps caused by ectopic expression of a germline RNA polymerase EGO-1 in the soma producing an increased RNAi effect (Wang et al., 2005).

Reduction of *isw-1* and *nurf-1* function suppressed abnormalities associated with defects in the germline-versus-soma cell-fate decision caused by *lin-15B(n744)* and *lin-35(n745)*, including the ectopic somatic expression of the germline-expressed protein PGL-1, the Tam phenotype (see Fig. S4 in the supplementary material) and the RNAi hypersensitivity phenotype (Fig. 3A-C; data not shown). However, the reduction of *isw-1* or *nurf-1* function did not cause a germline desilencing of expression from repetitive transgenes (data not shown), so both genes might not be required for mechanisms of transcriptional repression in the germline. Additionally, reduction of *isw-1* and *nurf-1* function suppressed the *mep-1* and *let-418* larval-arrest phenotypes (Fig. 3D), and somatic cells did not have a germline-like appearance in *isw-1; mep-1* or in *nurf-1; mep-1* double mutants (data not shown). These data indicate that not only are *isw-1* and *nurf-1* required for the synMuv vulval phenotype but also for the ectopic adoption of germline fates in the soma caused by loss of class B synMuv gene function. Therefore, the putative synMuv complexes and the NURF-like complex might antagonize the transcription of similar sets of target genes.

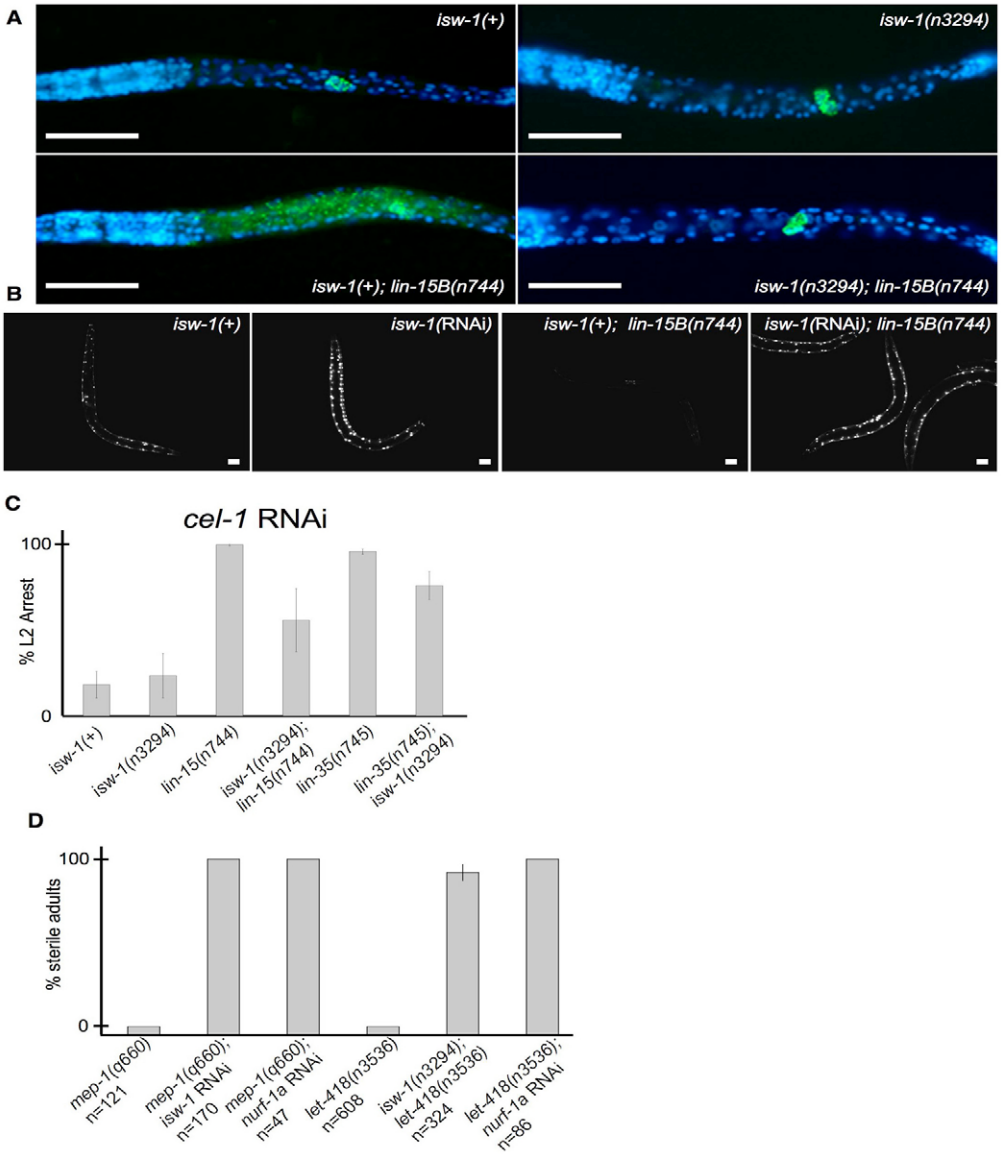

Fig. 3. Loss of *isw-1* function suppresses multiple cell-fate defects caused by mutations in class B synMuv genes. (**A**) *isw-1(n3294)* suppresses the ectopic expression of PGL-1 in the soma of L1 larvae in the class B synMuv mutant *lin-15B(n744)*. Anti-PGL-1 staining is shown in green and nuclei (4,6-diamidino-2-phenylindole (DAPI) staining) are shown in blue. Scale bars: 10 μm. (**B**) Loss of *isw-1* function suppresses the Tam phenotype of *lin-15B* animals and represses normal transgene expression. The *ccIs4251* reporter is a simple repetitive transgene that expresses GFP in the nuclei of body-wall muscles. From left to right, *ccIs4251* was expressed in the following backgrounds: wild type, *isw-1(RNAi)*, *lin-15B(n744)* and *isw-1(RNAi); lin-15B(n744)*. Scale bars:100 μm. A quantification of the Tam experiments is found in Fig. S4. (**C**) *isw-1* is required for the RNAi sensitivity of the class B synMuv mutant *lin-15B(n744)*. After exposure of animals to *cel-1* RNAi, the number of arrested L2 larvae was scored in at least three independent experiments. The average percent of L2 arrested larvae is shown. Error bars indicate standard deviations. (**D**) The activity of *isw-1* and *nurf-1* are required for the larval-lethal phenotypes of *mep-1(q660)* and *let-418(n3536)* mutants. The percent of sterile adults present at 25°C reflects the suppression of larval lethality. Error bars indicate standard deviations.

DISCUSSION

isw-1 and *nurf-1* antagonize the activities of at least the class B and C synMuv genes in the determination of *C. elegans* cell fates

The synMuv phenotype is caused by mutations in two genes in two different classes, and synMuv single mutants have a wild-type vulval phenotype (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989). The loss of *isw-1* or *nurf-1* function must antagonize a deficit in one or both of the synMuv genes in each suppressed strain to counteract the synMuv phenotype. Because the synMuv phenotypes of class AB, BC and AC double mutants were each suppressed by loss of *isw-1* or *nurf-1* function, the *C. elegans* NURF-like genes must antagonize the functions of at least two classes of synMuv genes. Defects caused by single class B mutants were suppressed by reduction of *isw-1* function (Fig. 3), and the vulval phenotype of the class C synMuv mutant *trr-1(n3712)* was suppressed (see Table S2 in the supplementary material). Thus, *isw-1* and *nurf-1* likely antagonize the activities of at least the class B and C synMuv genes. Because a functional Ras pathway is required for the synMuv phenotype, it is possible that the antagonism of the class B and C gene functions caused by reduction of *isw-1* or *nurf-1* function involves a downregulation of the Ras pathway.

isw-1 and *nurf-1* probably are not targets of synMuv-mediated transcriptional repression, as mRNA levels of each were not increased using semi-quantitative RT-PCR analysis of *lin-53; lin-15A* and *lin-35; lin-15A* mutants compared with the wild type (data not shown). Additionally, ISW-1 levels were not increased noticeably in *lin-53; lin-15A* and *lin-15AB* mutants, based on whole-mount immunofluorescence analysis (data not shown). Therefore, the synMuv genes probably do not act through the NURF-like genes to negatively regulate the vulval cell-fate decision.

ISW-1 and NURF-1 might be components of a NURF-like chromatin-remodeling complex involved in the *C. elegans* vulval cell-fate decision

C. elegans ISW-1 probably acts as part of a NURF-like complex and not as part of a CHRAC-like or ACF-like complex to antagonize the actions of the synMuv proteins, because inhibition

of *isw-1* or *nurf-1* but not inhibition of ACF or CHRAC ortholog gene functions suppressed the synMuv phenotype (Table 2). The *Drosophila* NURF complex is composed of four subunits: ISWI, NURF38, NURF55 and NURF301 (Tsukiyama and Wu, 1995). Because loss of the *C. elegans* homolog of NURF38, PYP-1, caused embryonic lethality (data not shown), we have not determined if it functions as a NURF-like complex component to antagonize the actions of the synMuv proteins. *Drosophila* NURF55 is similar to two proteins in *C. elegans*, LIN-53 and RBA-1 (72% and 53% identical, respectively). LIN-53 is a class B synMuv protein and 54% identical to its neighbor RBA-1. Strong reduction-of-function mutations in *lin-53* and *isw-1* cause opposite mutant phenotypes. Therefore, it is unlikely that LIN-53 and ISW-1 always act in the same complex. The LIN-53 homolog NURF55/RbAp48/CAF-1 is present in many chromatin-regulatory complexes, and it is possible that LIN-53 similarly acts in a number of *C. elegans* complexes, possibly both preventing and promoting the synMuv phenotype. If so, the role of LIN-53 in preventing the synMuv phenotype must be predominant, because loss-of-function mutations in *lin-53* cause a synMuv phenotype in combination with mutations in class A genes (Lu and Horvitz, 1998). Alternatively, RBA-1 might act with ISW-1 as part of a NURF-like complex. Because *rba-1(*RNAi*)* caused embryonic lethality (data not shown), we have not tested this possibility.

nurf-1, the *C. elegans* homolog of *Drosophila nurf301*, is predicted to encode at least five different proteins, each of which has some similarity to NURF301. Using deletion alleles and RNAi, we found that *nurf-1a* but not *nurf-1b*, *nurf-1c*, *nurf-1d* or *nurf-1e* was required to promote the synMuv phenotype. The region of NURF301 between the DDT domain (Doerks et al., 2001) and the C-terminal PHD fingers (Aasland et al., 1995) interacts with transcription factors required for recruitment of the NURF complex to target gene promoters (Xiao et al., 2001). The corresponding regions of NURF-1A, NURF-1B and NURF-1C differ in length and could mediate interactions with distinct sets of transcription factors to direct recruitment of the complex to different promoters. The NURF-1A region that presumably interacts with transcription factors might be responsible for recruitment of the NURF-like complex to promoters of genes required for the vulval cell-fate decision.

The functions of ISW-1 and NURF-1 might be involved in the generation of normal vulval cell fates

It is possible that the function of the putative *C. elegans* NURF-like complex is required only for the generation of ectopic vulval cell fates, e.g. in synMuv mutants, but is not involved in normal vulval development. For example, when the inhibitory actions of the synMuv proteins are absent or when the activity of the Ras pathway is increased, the NURF-like complex might promote the specification of ectopic vulval cell fates. However, we observed that *isw-1* is required not only for the Tam phenotype of class B synMuv mutants (Fig. 3B) but also for a basal level of repression of expression from the *ccIs4251* GFP reporter (see Fig. S4 in the supplementary material). This observation suggests that *isw-1* is required not only in the absence of synMuv activity but also in a wild-type synMuv background to promote expression of genes repressed by the synMuv proteins. By analogy, we propose that the putative NURF-like complex helps promote the normal generation of vulval cell fates in a wild-type synMuv background.

The *C. elegans* NURF-like complex acts antagonistically to complexes similar to Myb-MuvB/dREAM, NuRD and Tip60/NuA4 to control transcription

The *Drosophila* NURF complex slides nucleosomes along the DNA to allow access for transcription factors to bind target sequences in vitro (Hamiche et al., 1999). Both ISWI and NURF301 are required for the transcription of Hox and heat-shock genes in vivo (Deuring et al., 2000; Badenhorst et al., 2002). Therefore, the NURF complex has been hypothesized to be involved in transcriptional activation. The homologs of many class B synMuv proteins are components of at least two complexes, Myb-MuvB/dREAM and NuRD, involved in transcriptional repression (Tong et al., 1998; Xue et al., 1998; Korenjak et al., 2004; Lewis et al., 2004). Studies of *Drosophila* and mammalian cells argue that the NURF complex and the Myb-MuvB/dREAM and NuRD complexes have opposite effects on transcription.

The vulval cell-fate decision in *C. elegans* demonstrates the biological consequences of the opposing effects of the Myb-MuvB/dREAM and NuRD and the NURF chromatin-remodeling activities. We propose that a complex containing both ISW-1 and NURF-1 antagonizes one or more synMuv protein complexes in the transcriptional control of the vulval cell-fate decision. One hypothesis is that loss of transcriptional repression by the synMuv proteins causes a Muv phenotype, as a result of the increased transcription of genes that promote the vulval cell-fate decision. The NURF-like complex might be required for this ectopic expression of synMuv target genes. Alternatively, the NURF-like complex might act at targets distinct from those that are misregulated in synMuv mutants, and transcription of these genes would antagonize the activities of the synMuv proteins. The identification of the transcriptional targets of the synMuv proteins and of the NURF-like complex should help differentiate between these two hypotheses. The *Drosophila* Myb-MuvB complex co-purified with sub-stoichiometric amounts of NURF complex members. The actions of and requirements for the NURF complex components for Myb-MuvB function were not investigated (Lewis et al., 2004). Perhaps NURF-like complexes bind Myb-MuvB-like complexes to directly inhibit activities of these complexes.

The antagonism of the *lin-35* Rb and *let-60* Ras mutant phenotypes by partial loss of *isw-1* ISWI function suggests a possible approach to cancer therapy

The functional antagonism between a NURF-like complex and synMuv repressive complexes and/or activation of the Ras pathway could be conserved in humans and be important for human cancer. The loss of Rb function is associated with the majority of human carcinomas (Adams and Kaelin, 1998), and methods to inhibit the defects of Rb-deficient cells should be beneficial as cancer treatment strategies. Additionally oncogenic forms of human Ras are involved in many cancers, especially cancers of the lung (Minamoto et al., 2000). Because a reduction of *isw-1* ISWI function can suppress defects associated with a complete loss of *lin-35* Rb or activation of *let-60* Ras in *C. elegans*, inhibition of the human ISW-1 homolog (SNF2H; SMARCA5 – Human Gene Nomenclature Database) might suppress the effects of Rb loss or of oncogenic Ras in human cells and hence reduce or eliminate the consequences of oncogenic mutations. SNF2H is a chromatin-remodeling enzyme (Okabe et al., 1992; Aihara et al., 1998) and might be a reasonable target for therapeutic intervention.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/14/2695/DC1

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