Recruitment of *C. elegans* dosage compensation proteins for gene-specific versus chromosome-wide repression

Stephanie A. Yonker and Barbara J. Meyer*

Howard Hughes Medical Institute, Department of Molecular and Cell Biology, 16 Barker Hall, University of California, Berkeley, CA 94720-3204, USA

*Author for correspondence (e-mail: bjmeyer@uclink.berkeley.edu)

Accepted 26 September 2003

Development 130, 6519-6532
Published by The Company of Biologists 2003
doi:10.1242/dev.00886

Summary

In *C. elegans*, an X-chromosome-wide regulatory process compensates for the difference in X-linked gene dose between males (XO) and hermaphrodites (XX) by equalizing levels of X-chromosome transcripts between the sexes. To achieve dosage compensation, a large protein complex is targeted to the X chromosomes of hermaphrodites to reduce their expression by half. This repression complex is also targeted to a single autosomal gene, *her-1*. By silencing this male-specific gene, the complex induces hermaphrodite sexual development. Our analysis of the atypical dosage compensation gene *dpy-21* revealed the first molecular differences in the complex that achieves gene-specific versus chromosome-wide repression. *dpy-21* mutations, shown here to be null, cause elevated X-linked gene expression in XX animals, but unlike mutations in other dosage compensation genes, they do not cause extensive XX-specific lethality or disrupt the stability or targeting of the dosage compensation complex to X. Nonetheless, DPY-21 is a member of the dosage compensation complex and localizes to X chromosomes in a hermaphrodite-specific manner. However, DPY-21 is the first member of the dosage compensation complex that does not also associate with *her-1*. In addition to a difference in the composition of the complex at *her-1* versus X, we also found differences in the targeting of the complex to these sites. Within the complex, SDC-2 plays the lead role in recognizing X-chromosome targets, while SDC-3 plays the lead in recognizing *her-1* targets.

Key words: Dosage compensation, Sex determination, Gene regulation, *C. elegans*, DPY-21, SDC-2, SDC-3, *her-1*

Introduction

Sex is determined in many organisms by an X-chromosome counting mechanism that distinguishes one X chromosome from two (e.g. XO male/XX female in nematodes) or by the presence of a specific sex chromosome, such as the Y chromosome (e.g. XY male/XX female in mammals) (Meller and Kuroda, 2002; Meyer, 2000). In these organisms, a chromosome-wide regulatory process called dosage compensation copes with the difference in X-linked gene dose between males and females by equalizing levels of X-chromosome transcripts (Meller and Kuroda, 2002; Meyer, 2000). To achieve dosage compensation, specialized complexes are targeted to the X chromosome(s) of one sex to modulate X transcript levels. This sex-specific, chromosome-wide regulation is superimposed upon the regulation of individual X-linked genes that occurs in both sexes. Failure to accomplish dosage compensation causes sex-specific lethality. In the nematode *C. elegans*, dosage compensation is achieved by a protein complex that binds to both X chromosomes of hermaphrodites to reduce their transcript levels by half (Meyer, 2000). Remarkably, the dosage compensation complex is similar to the evolutionarily conserved 13S condensin complex required for mitotic and meiotic chromosome resolution and compaction, implying the recruitment of ancient chromosome segregation proteins to the new task of regulating gene expression (Chuang et al., 1994; Hagstrom et al., 2002; Lieb et al., 1998; Lieb et al., 1996).

Typically, protein complexes that regulate gene expression across entire chromosomes or subchromosomal domains do not function as specific regulators of individual genes. However, the *C. elegans* dosage compensation complex is unusual in this regard. Not only does the complex repress expression of X chromosomes by twofold, it represses transcription of the autosomal sex determination gene *her-1* by 20-fold (Chu et al., 2002; Dawes et al., 1999). This contrast led us to investigate how a protein complex achieves uniformly weak repression of numerous genes in one context and strong repression of a specific gene in another. The work presented here on the dosage compensation gene *dpy-21* reveals distinctions in the composition and recruitment of the complexes that achieve these two levels of repression.

Prior research showed that sex determination and dosage compensation in *C. elegans* are coordinately controlled in response to the X-chromosome counting mechanism (DeLong et al., 1993; Klein and Meyer, 1993; Miller et al., 1988; Nusbaum and Meyer, 1989; Rhind et al., 1995; Villeneuve and Meyer, 1987). In XX embryos, SDC-2 is the pivotal factor that initiates dosage compensation (Dawes et al., 1999). It is the only dosage compensation protein known to be expressed exclusively in hermaphrodites, and it can localize to X chromosomes independently of other dosage compensation

proteins. SDC-2 acts together with SDC-1 and SDC-3 to trigger assembly of the condensin-like dosage compensation proteins (DPY-26, DPY-27 and MIX-1) onto hermaphrodite X chromosomes to reduce gene expression by half. In fact, ectopic expression of SDC-2 in males is sufficient to trigger assembly of this complex onto the single X chromosome, causing death from inappropriately low X-linked gene expression. The SDC complex also induces hermaphrodite sexual development in XX embryos by associating with the promoter of the male sex-determining gene *her-1* to repress its transcription (Chu et al., 2002; Dawes et al., 1999). Again, SDC proteins recruit DPY-26, DPY-27 and MIX-1, this time to *her-1* regulatory regions (Chu et al., 2002). This localization, together with the observation that *dpy* mutations affect sexual fate in specific genetic backgrounds, suggests that DPY proteins may act directly with SDC proteins to repress *her-1*.

dpy-21 is also required for dosage compensation; however, *dpy-21* differs significantly from other dosage compensation genes. Like mutations in other dosage compensation genes, *dpy-21* mutations cause elevated X-linked gene expression and morphological phenotypes dependent upon X-chromosome dose: XO animals appear wild type, while XX animals are dumpy and egg-laying defective (L. DeLong, PhD thesis, Massachusetts Institute of Technology, 1990) (DeLong et al., 1987; Hodgkin, 1983; Meneely and Wood, 1984; Meneely and Wood, 1987; Meyer and Casson, 1986; Plenefisch et al., 1989). But unlike other dosage compensation mutations, which disrupt the stability or X-localization of the dosage compensation complex, causing extensive XX-specific lethality, *dpy-21* mutations, shown here to be null, cause no embryonic lethality and only infrequent larval lethality (Chuang et al., 1996; Lieb et al., 1998; Lieb et al., 1996; Plenefisch et al., 1989). Consistent with the weak dosage compensation phenotype, *dpy-21* null mutations cause no obvious defect in the assembly or localization of the dosage compensation complex (Chuang et al., 1996; Davis and Meyer, 1997; Lieb et al., 1998; Lieb et al., 1996). We place DPY-21 in the molecular pathway of dosage compensation.

We show that DPY-21 is a member of the dosage compensation complex and localizes to X chromosomes in a sex-specific manner. DPY-21 is the first member of the dosage compensation complex that does not also localize to *her-1*. Moreover, we show that recognition of *her-1* target DNA is initiated by a different SDC protein from that responsible for initial recognition of X-chromosome targets. These findings represent the first molecular differences in the repression complexes that achieve gene-specific versus chromosome-wide regulation.

Materials and methods

Identification of ESTs

dpy-21 was mapped with sequence tagged site (STS) polymorphisms to a 500 kb interval of chromosome V located between cosmids K03D8 and C48B12 and covered by YACs Y59A8 and Y94A7. To identify cDNA clones from this interval, the Data Base of Expressed Sequence Tags (dbEST from Y. Kohara, National Institute of Genetics, Mishima, Japan) was searched with the partially assembled YAC sequences using the Basic Local Alignment Search Tool (BLAST). The Smallest Sum of Probability score [P(N)] was calculated between the sequence of each EST and the YAC sequences to determine the probability that the two sequences had the same overall similarity by chance. ESTs with a P(N) score less than e^{-10} were chosen for further study, resulting in 108 ESTs. EST candidates were grouped into 30 families based on predicted amino acid sequence similarity to related proteins. This approach prevented the analysis of multiple ESTs from the same ORF. Six out of 30 cDNA families were judged unlikely to participate in dosage compensation and were therefore eliminated from preliminary consideration.

RNA isolation for RNAi experiments

Plasmids were excised from cDNA-containing phagemids (provided Y. Kohara), linearized with either *Kpn*I or *Not*I, and used as template for either Ribomax (Promega) T3 or T7 transcription reactions. Single-stranded RNAs (ssRNAs) were injected at a concentration of 1 mg/ml.

Genetic assays to clone *dpy-21*

ssRNA was tested for its ability to mimic a *dpy-21* mutation in two genetic assays. In the first assay, single-stranded RNA (ssRNA) corresponding to each EST family was tested for its ability to rescue the XO-specific lethality caused by a *xol-1* mutation. *xol-1* mutations inappropriately activate the hermaphrodite program of dosage compensation in XO males, causing male lethality from reduced X-linked gene expression (Miller et al., 1988). Mutations in *dpy-21* suppress the XO lethality by disrupting dosage compensation. ssRNAs were injected into a *him-5(e1485); xol-1(y9)* strain and the progeny were examined for males. The *him-5* mutation causes X-chromosome non-disjunction and was included in the *xol-1* strain to generate XO animals. Partial rescue of male lethality was observed with ssRNA corresponding to cDNA clones yk132a2 and yk278g2.

In the second assay, the strongest *xol-1* suppressor (ssRNA to yk132a2) was tested for its ability to suppress the masculinization caused by *sdc-3*(Tra). Only 1% of *sdc-3(y52*Tra*)* XX homozygous animals from *sdc-3(y52*Tra*)*/+ mothers are hermaphrodites at 20°C, but 65% of *sdc-3(y52*Tra*) dpy-21(e428)* XX animals from *sdc-3(y52*Tra*) dpy-21(e428)*/++ mothers are hermaphrodites (DeLong et al., 1993). To test yk132a2, ssRNA was injected into *sdc-3(y52*Tra*) unc-76(e911)/sdc-3(y128*Dpy*)* animals and the percentage of Unc Tra male and Unc hermaphrodite progeny was determined. Thirty-five percent of the Unc progeny (n=220) were hermaphrodites, indicating suppression similar to a *dpy-21* mutation. Together, these assays indicate that yk132a2 is likely to represent *dpy-21*.

DNA sequence analysis of yk132a2 and yk278g2 showed that both cDNAs represented the same gene (ORF Y59A8b.1), a conclusion previously obscured by the fact that the cDNAs were incomplete and differed in their 5′ and 3′ ends. The true 5′ end of Y59A8b.1 was obtained using PCR with a primer to the *C. elegans* trans-splice leader SL1 and an internal primer.

RT-PCR of *dpy-21*

Total *C. elegans* RNA was isolated as described (DeLong et al., 1993). mRNA was purified using Oligotex (Qiagen). *dpy-21* cDNA was made by reverse transcription using primers DPY-21.51 (5′ GCAAATAGGGGTACTCCATTG 3′) and SuperScriptII (Invitrogen). To amplify *dpy-21*, first-round nested PCR used primers DPY-21.52 (5′ GATCTCATCGGGTAAAGGATTC 3′) and NOTSL1, which includes the SL1 splice leader and a *Not*I restriction site. Second-round nested PCR used primers DPY-21.53 (5′ GTGTATGAA-GCGAAGAACTTCG 3′) and NOTSL1.

Sequence analysis of *dpy-21* mutants

Molecular lesions in the *dpy-21* mutants were identified by sequence analysis of genomic DNA (*e428* and *y59*) or RT-PCR products (*e459*, *y43*, *y47*, *y188*ts and *y150*ts). Sequence analysis was performed on three different DNA preparations for each mutation. The DNA changes were as follows: *y428*, CAG to TAG at codon 394; *y59*, CAG to TAG at codon 417; *e459*, GGA to GAA at codon 1291; *y150*ts, CTC to TTC at codon 1383; *y88*ts, AAG to GAG at codon 1396; *y47*,

CAG to TAG at codon 1423. *dpy-21(y43)* has both a silent change at bp 4290 and a 134 bp deletion that removes the intron/exon 8 boundary. A splice site 87 base pairs upstream of the normal splice site is used, resulting in an in-frame deletion of 29 amino acids, as shown by sequence analysis of RT-PCR products.

Stage-specific northern

RNA was made from wild-type embryos, L1, L2, L3 and L4 larvae, and non-gravid adults according to a protocol by G. Csankovszki. Trizol (10 ml, Invitrogen) was added to 2-3 ml of packed animals and the mixture homogenized using a polytron. The aqueous sample was subjected to three sequential extractions using 2 ml chloroform, then an equal volume of phenol and 0.2 volumes of chloroform, and, finally, an equal volume of chloroform. For each extraction, the phases were separated by centrifugation at 9000 ***g*** for 15 minutes at 4°C. The aqueous layer was mixed with 0.5 volumes of 2-propanol and incubated at room temperature for 10 minutes. The RNA was pelleted at 9000 ***g*** for 15 minutes at 4°C and washed with 70% ethanol. mRNA was purified from total RNA using Oligotex (Qiagen). Poly-A RNA (5 μg) was separated by gel electrophoresis, and northern analysis was performed (Wutz and Jaenisch, 2000) using random-primed probes transcribed from nucleotides 1-3347 of *dpy-21*.

Antibodies

Rabbit anti-DPY-21 antibodies were raised against a fusion protein composed of a GST tag and DPY-21 amino acids 1-173 that had been expressed from vector pGEX-5X-2 (Amersham) and purified using Glutathione Sepharose 4B (Amersham). Affinity purification of the anti-DPY-21 antibodies was performed as described (Davis and Meyer, 1997), using a 6×-His-tagged fusion protein that was made to the same DPY-21 region, expressed from vector pRSETA (Invitrogen), purified with Ni-NTA spin (Qiagen) and coupled to 6×Reacti-Gel (Pierce).

Rabbit anti-DPY-21 antibodies were also raised against a fusion protein composed of DPY-21 amino acids 467-1102, a GST tag at the N terminus and a 6×-His tag at the C terminus. The plasmid used to express the antigen was constructed by subcloning a RT-PCR product corresponding to nucleotides 1533-3460 of the *dpy-21* transcript into pGEX-5X-2 vector that had been modified by adding 6 histidines prior to the stop codon. Affinity purification of these anti-DPY-21 antibodies was performed (Davis and Meyer, 1997) using the 6×-His-tagged fusion protein coupled to 6×Reacti-Gel (Pierce) with one modification. The serum was first passed through a GST-only column (gift of R. Chan) to remove antibodies against GST, and the flow-through was collected for antigen-specific purification.

SDC-3 was detected in embryos and in *her-1* array experiments using affinity-purified rat polyclonal antibodies raised against amino acids 1067-1340 (from C. Tsai).

Extract preparation

Extracts were prepared by boiling wild-type and mutant embryos in four volumes of SDS-PAGE loading buffer (100 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 7.5 M Urea, and 0.1 M DTT) for 10 minutes. Insoluble debris was removed by centrifugation at 9300 ***g*** for 1 minute. SDS-PAGE (Novex) and immunoblotting (Bio-Rad) using chemiluminescent detection (ECL, Amersham) were performed with the extract.

Immunoprecipitation experiments

Lysates were prepared by sonicating embryos in chromatin buffer (200 mM sucrose, 5 mM Tris, pH 7.5, 80 mM NaCl, 1 mM $CaCl_2$) supplemented with a protease inhibitor cocktail (Calbiochem). Lysates were titrated to 5 mM EDTA and allowed to rock at 4°C for 20 minutes. Cellular debris was cleared by centrifugation at 4500 ***g*** for 5 minutes at 4°C. The supernatant was incubated with 5 μg primary antibody for 4 hours at 4°C and then incubated with Protein A Sepharose beads for 1 hour. Immunocomplexes bound to the Protein A Sepharose beads were washed with CHIP buffer (50 mM HEPES, pH 7.6, 140 mM KCl, 1 mM EDTA, and 0.5% NP-40). Proteins were eluted by boiling the beads in an SDS dye (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 0.1 M DTT). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting.

Immunostaining of embryos and gut cells

Embryos were collected, fixed, and stained as described (Chuang et al., 1994; Davis and Meyer, 1997), except that embryos were fixed at room temperature for 15 minutes, and all washes were in PBST (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.5% Triton X-100 and 1 mM EDTA, pH 8.0). Gut cells were stained as described (Howe et al., 2001). For confocal microscopy, animals were mounted in Vectashield (Vector Laboratories) containing 1 μg/ml of DAPI. Laser confocal microscopy was performed on a Leica TCS-NT confocal microscope. Over 100 embryos were analyzed in most immunostaining experiments.

X-chromosome fluorescence in situ hybridization (FISH) and anti-DPY-21 staining in embryos

The X-chromosome FISH protocol was developed in collaboration with G. Csankovszki and T. Wu based on the FISH protocol previously described (Dernburg and Sedat, 1998). Gravid hermaphrodites were removed from plates and washed with M9 (8.5 mM NaCl, 41 mM Na_2HPO_4, 8.5 mM KH_2PO_4 and 18 mM NH_4Cl) and treated with hypochlorite to obtain embryos. After several washes with M9, an equal volume of embryos was added to sperm salts (50 mM PIPES, pH 7.0, 25 mM KCl, 1 mM $MgSO_4$, 45 mM NaCl and 2 mM $CaCl_2$) containing 3% paraformaldehyde (Electron Microscopy Sciences) on a poly-lysine coated slide and incubated in a humid chamber for 5 minutes. The slide was freeze cracked on dry ice, placed in 95% ethanol for 1 minute and washed with PBST. Subsequently, the slide was dehydrated for 2 minutes each in 70% ethanol, 80% ethanol, 95% ethanol and 100% ethanol, then allowed to air dry. X-chromosome fluorescent probe (10 μl) made by random primed labeling (Promega) of X-specific YAC DNA (gift of G. Csankovszki) was added. The slide was denatured at 95°C for 3 minutes, then incubated overnight in a humid chamber at 37°C. The slide was washed three times for 5 minutes each at 39°C with 2×SSC (0.3 M NaCl and 30 mM $Na_3C_6H_5O_7$) in 50% formamide, three times for 5 minutes each at 39°C with 2×SSC, and once for 10 minutes at 39°C with 1×SSC. The slide was rinsed in PBST and antibody staining was performed as described for immunostaining of adult germline and gut cells.

Localization of DPY-21 was analyzed in dosage compensation mutants. Dosage compensation mutations that cause XX-specific lethality were maintained in XO strains for which the hermaphrodite mode of sex determination and dosage compensation had been switched on by a *xol-1* mutation. XO-specific lethality caused by the *xol-1* mutation was suppressed by the dosage compensation mutation being assayed. For *sdc-2* and *sdc-3* mutant strains, a *her-1* mutation was included to allow the XO animals to develop as hermaphrodites. The genotypes of strains were:

sdc-1(n485);
dpy-26(n199) unc-30(e191) IV; lon-2(e678) xol-1(y9) V;
unc-32(e189) dpy-27(y167) III; flu-2(e1003) xol-1(y9) V;
dpy-28(s939) III; him-5(e1490); xol-1(y9) V;
her-1(hv1y101) V; xol-1(y9) sdc-2(y74) unc-9(e101) X; and
her-1(e1520) sdc-3(y126) V; xol-1(y9) X.

The dosage compensation mutations used in the staining experiments were molecular or genetic nulls.

her-1 array experiments

Except for *her-1* array strains carrying either an *sdc-2* or *sdc-3* mutation, previously established *her-1* array and control lines were used to examine DPY-21 localization (Chu et al., 2002; Dawes et al.,

1999). All *her-1* arrays contained the *her-1* region indicated and plasmids encoding LacI::GFP and *Lac O* repeats. Animals were heat-shocked at 34°C for 30 minutes and allowed to recover at room temperature for 30 minutes to induce production of the LacI::GFP fusion protein, which is controlled by a heat-shock promoter.

Results

dpy-21 mutations disrupt a novel, conserved protein

We cloned *dpy-21* to determine its molecular identity and its role in the regulatory hierarchy that controls dosage compensation. *dpy-21* was first mapped with sequence tagged site (STS) polymorphisms to a 500 kb interval of chromosome V. An RNAi strategy involving two genetic assays was then used to identify a candidate *dpy-21* EST from the pool of ESTs correlated with this interval (Materials and methods). The EST yk132a2 emerged as the most likely candidate to represent *dpy-21*. The gene structure and complete DNA sequence of the ORF (Y59A8b.1) that corresponded to yk132a2 were determined.

The detection of molecular changes in ORF Y59A8b.1 from several *dpy-21* mutant alleles proved that *dpy-21* had been correctly identified (Fig. 1A). *dpy-21(e428)* is a nonsense mutation predicted to truncate DPY-21 at amino acid 394. This mutation causes the most severe *dpy-21* mutant phenotype: 17% larval lethality and all adult survivors are dumpy (Dpy) and egg-laying defective (Egl). *dpy-21(y59*am*)* and *dpy-21(y47*am*)* are also nonsense mutations, predicted to terminate translation at codons 417 and 1396, respectively, and both can be suppressed by the amber mutant tRNA suppressor *sup-7*. *y47* and *e428* cause very similar phenotypes. *dpy-21(e459)* and the temperature-sensitive mutations *dpy-21(y88*ts*)* and *dpy-21*(*y150*ts) all have missense mutations (Fig. 1A). *y88* causes the weakest phenotype: no lethality and all adults are Dpy and Egl. Finally, *dpy-21(y43)* has a 134 bp deletion that results in an in-frame deletion of 29 amino acids.

Previous genetic analysis of the 24 *dpy-21* mutations was inconclusive regarding the null phenotype, as the chromosomal deficiencies that eliminate *dpy-21* are haploinsufficient, causing lethality by themselves (L. DeLong, PhD thesis, Massachusetts Institute of Technology, 1990). The identification of nonsense mutations and their effect on DPY-21 protein levels (see below), together with our finding that *dpy-21* RNAi treatment of *dpy-21(e428)* mutants does not enhance the mutant phenotype, strongly suggests that the weakness of the *dpy-21* dosage compensation phenotype is not caused by incomplete disruption of the gene. Rather, *dpy-21* is genuinely less essential than other dosage compensation genes.

The *dpy-21* gene includes 11 introns and is predicted to encode a 5629 bp transcript. DPY-21 appears to be a novel, conserved protein of 1641 amino acids with a proline-rich N terminus. No other motifs could be identified based on sequence. However, homologs of DPY-21 share a unique, evolutionarily conserved C-terminal domain (amino acids 1230-1620) (Fig. 2). The biochemical functions of the proteins in this family are unknown.

Regulation of *dpy-21* transcript levels during development

Transcript levels of most dosage compensation genes peak during embryogenesis and decline during larval stages

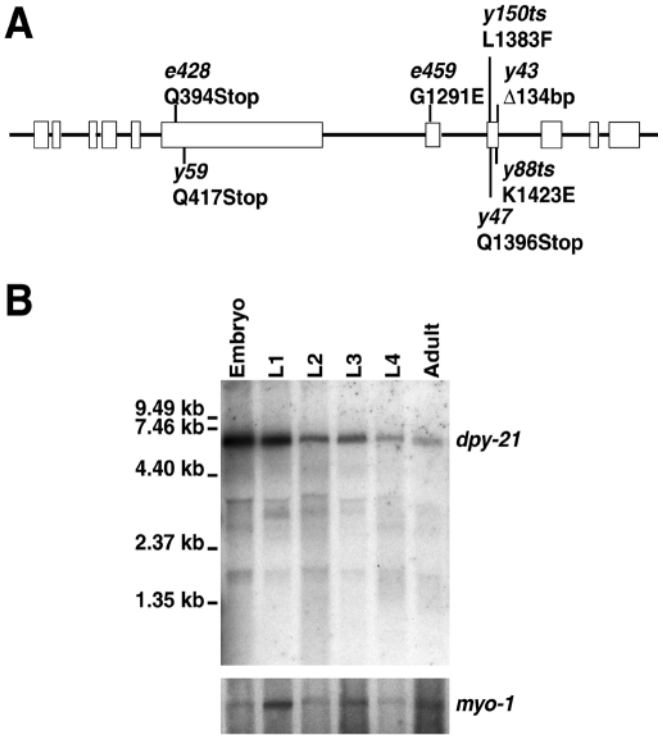


Fig. 1. Map of *dpy-21* molecular lesions and levels of *dpy-21* transcripts throughout development. (A) The intron-exon gene structure of *dpy-21*. The molecular changes for seven *dpy-21* mutant alleles are indicated. Genetically, *dpy-21(e428)* is the most severe mutation, causing 17% larval lethality and dumpy, egg-laying defective adult survivors, some with a protruding vulva. (B) Northern blot of mRNA isolated from wild-type embryos, L1, L2, L3 and L4 larvae, and young adults without embryos. The blot was hybridized with a probe to the first 3347 nucleotides of the *dpy-21* transcript and a *myo-1* probe to measure pharyngeal myosin transcript levels as a loading control. The *dpy-21* transcript is expressed throughout development, with the highest transcript levels occurring during embryogenesis.

(Chuang et al., 1994; Klein and Meyer, 1993). To determine the developmental profile of *dpy-21* transcripts, mRNA prepared from animals in each development stage was blotted and hybridized with a probe specific to the first 3347 nucleotides of *dpy-21* (Fig. 1B). The probe detected a single *dpy-21* transcript that is expressed throughout development and migrates at ~5700 bp, consistent with the predicted transcript size of 5629 bp. The *dpy-21* transcript levels appeared highest during embryogenesis after normalization to the *myo-1* (pharyngeal myosin) control transcript. This expression pattern resembles that of other dosage compensation genes.

DPY-21 interacts biochemically with components of the dosage compensation complex

All previously identified dosage compensation proteins form a complex that localizes to hermaphrodite X chromosomes and represses their gene expression (Chu et al., 2002; Chuang et al., 1996; Davis and Meyer, 1997; Dawes et al., 1999; Lieb et al., 1998; Lieb et al., 1996). To determine whether DPY-21 is a member of the dosage compensation complex, antibodies were raised to two different regions of DPY-21, the first 173

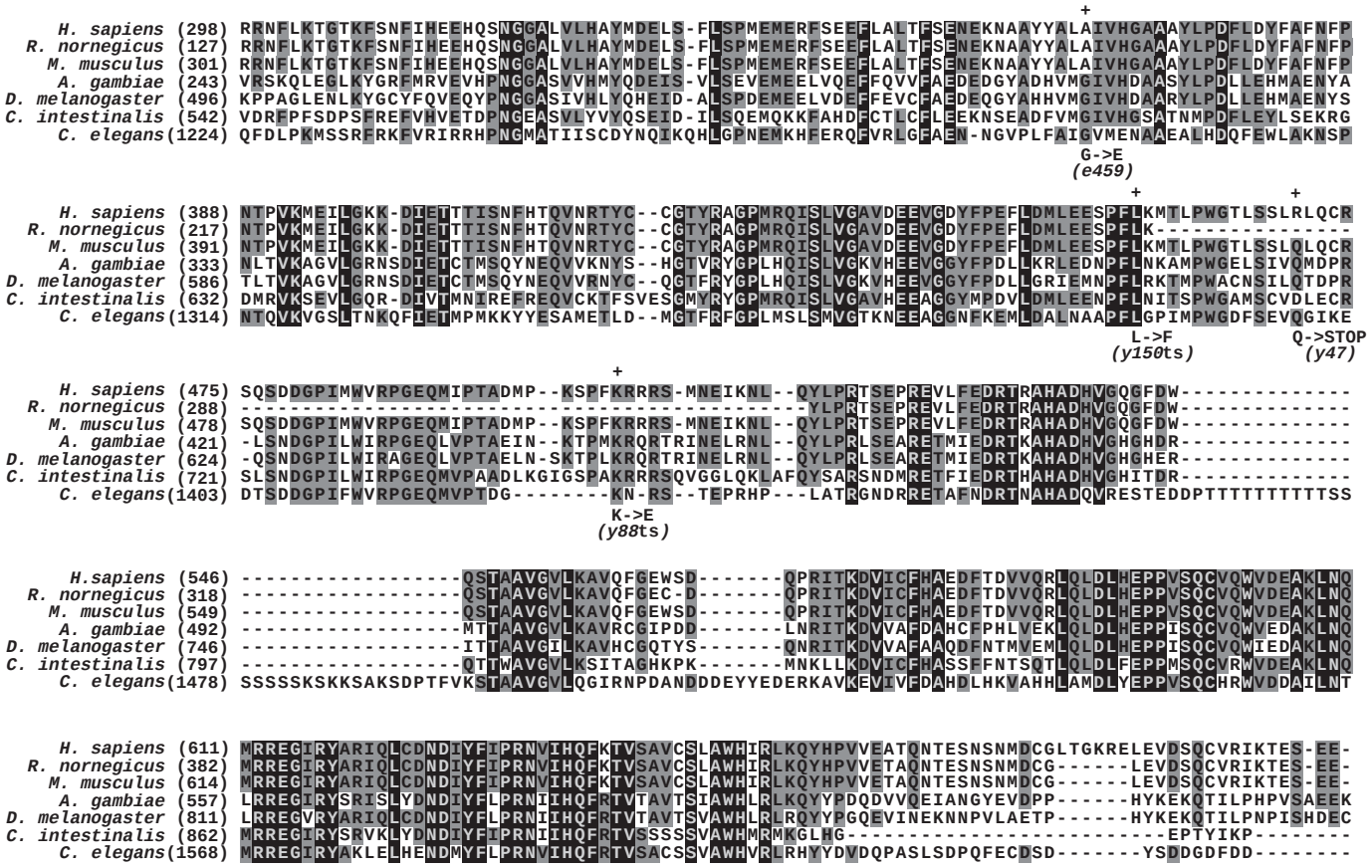


Fig. 2. The C-terminal domain of DPY-21 is conserved between species. In the alignment of the C terminus (amino acids 1224-1641), black indicates sequence identity, and gray represents sequence similarity. DPY-21 contains no identifiable motifs. However, the C-terminal region of DPY-21 appears to be conserved throughout evolution. No other significant similarity was found between DPY-21 and the putative homologs, as they are rendered in current data bases. The function of these DPY-21 homologs has not been determined. Locations of *dpy-21* mutations are indicated by the + symbol.

amino acids and an internal peptide (amino acids 467-1102) (Materials and methods). Two findings indicate that these antibodies are specific to DPY-21. First, both antibodies recognized a 210 kDa protein in extracts from wild-type gravid adults that was not detectable in extracts from either *dpy-21(e428)* or *dpy-21(y59)* mutant gravid adults (Fig. 3A and data not shown). The apparent molecular weight of this protein is slightly larger than the predicted size of 185 kDa. A control assessing levels of SMC-1, a chromosome cohesion protein, demonstrated that the *dpy-21(e428)* mutant and wild-type extracts had comparable levels of proteins (Fig. 3A). Second, the N-terminal antibody detected a protein of 60 kDa in the *dpy-21(e428)* mutant extract, consistent with the size of a truncated DPY-21 product predicted from the location of the nonsense mutation at codon 394 (Fig. 3A). The truncated protein was detected variably.

To test whether DPY-21 associates physically with other members of the dosage compensation complex, immunoprecipitation reactions were performed using antibodies to several dosage compensation proteins. DPY-21 antibodies co-immunoprecipitated the dosage compensation protein SDC-3 (Fig. 3B), and antibodies to the dosage compensation protein DPY-27 co-immunoprecipitated DPY-21 (Fig. 3C). Three controls showed that the precipitation reactions were specific, allowing us to conclude that DPY-21 interacts biochemically with dosage compensation proteins. Mock reactions without antibodies (Fig. 3B), reactions with preimmune sera (Fig. 3B,C), and reactions with antibodies against CBP-1, a DNA-associated CREB-binding protein (Fig. 3C), failed to precipitate dosage compensation proteins.

Although DPY-21 interacts with other dosage compensation proteins, two differences were noticed in the behavior of DPY-21 compared with the other proteins. First, the immunoprecipitation reactions were not reciprocal in that DPY-27 antibodies immunoprecipitated DPY-21, but DPY-21 antibodies did not precipitate DPY-27. Second, antibodies to the dosage compensation protein DPY-26 precipitated DPY-21 only weakly, even though it precipitates DPY-27 and MIX-1 robustly. Although we do not know the reasons for these differences, we speculate that the antibodies might disrupt interactions between DPY-21 and other dosage compensation proteins or that the association between DPY-21 and the other proteins might not be as strong as that of other components. These observations suggest that the function of DPY-21 within the complex may be different from that of the other proteins.

DPY-21 localizes specifically to both X chromosomes of hermaphrodites

If DPY-21 functions as a member of the dosage compensation complex in vivo, as predicted by the biochemical experiments,

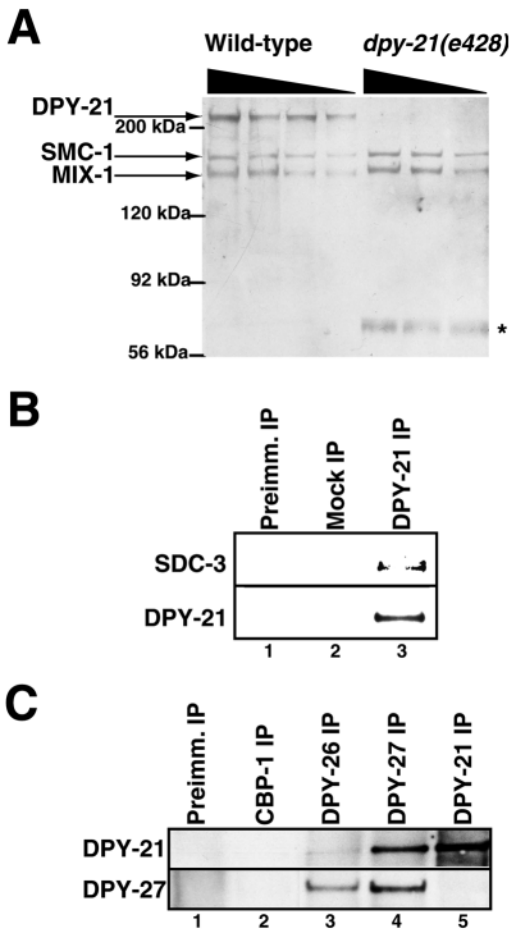


Fig. 3. DPY-21 associates physically with components of the dosage compensation complex. (A) Western blot of extracts from wild-type or *dpy-21(e428)* mutant gravid hermaphrodites serially diluted by 1.3-fold and probed with N-terminal antibodies to DPY-21 and antibodies to the loading control SMC-1, a protein involved in chromosome cohesion. Full-length DPY-21 (~210 kDa) was present in extracts from wild-type but not *dpy-21* mutant animals. A ~60 kDa protein was variably detected in the *dpy-21* mutant extract (asterisk). The apparent molecular weight of this protein is slightly larger than the 43 kDa protein predicted from the location of the *e428* nonsense mutation at codon 394. The blot was also probed with antibodies to MIX-1, a protein involved in dosage compensation and chromosome condensation. Equivalent levels of MIX-1 in both mutant and wild-type extracts provide one example that *dpy-21* mutations do not alter the stability of other dosage compensation proteins. (B) DPY-21 antibodies specifically precipitated SDC-3 from wild-type embryonic extracts (lane 3). SDC-3 was not immunoprecipitated by the preimmune sera (lane 1) or in a mock immunoprecipitation reaction that lacked antibodies (lane 2), showing specificity of the IP reactions. (C) DPY-27 antibodies strongly precipitated DPY-21 (lane 4). The precipitation of DPY-21 was specific, given that DPY-21 was not precipitated by the preimmune sera (lane 1) or antibodies to CBP-1, a DNA-associated CREB-binding protein (lane 2). DPY-21 antibodies failed to precipitate DPY-27 (lane 5), and DPY-26 antibodies only weakly precipitated DPY-21 (lane 3). These immunoprecipitation experiments indicate that DPY-21 interacts biochemically with other dosage compensation components but its association with the complex is probably not as robust as that of other members.

DPY-21 should localize to X chromosomes of XX embryos at or beyond the 40-cell stage, when SDC-2 recruits dosage compensation proteins to X. Immunofluorescence experiments using DPY-21 antibodies showed DPY-21 to be diffusely localized in nuclei of XX embryos (<40 cells) that had not yet activated dosage compensation (Fig. 4A). In embryos with greater than 40 cells, DPY-21 formed punctate, subnuclear foci that coincided with the X-localized SDC-3 foci (Fig. 4B). We showed directly that the DPY-21 foci co-localized with X chromosomes by probing simultaneously with DPY-21 antibodies and X-chromosome-specific DNA probes that were visualized by fluorescence in situ hybridization (FISH) (Fig. 5A). DPY-21 maintains its localization to X chromosomes throughout *C. elegans* development, like other dosage compensation proteins, as indicated by its presence on X chromosomes of adult gut nuclei (Fig. 4D). The in vivo specificity of both DPY-21 antibodies was confirmed by the absence of staining in the two *dpy-21* mutant strains homozygous for early nonsense mutations, *dpy-21(e428*) (Fig. 4C and data not shown) or *dpy-21(y59)* (data not shown). These results establish that DPY-21 is recruited to the X chromosomes of hermaphrodites with other members of the dosage compensation complex at the onset of dosage compensation.

DPY-21 is expressed in males, but fails to localize to the single X chromosome

dpy-21 mutations disrupt X-linked gene expression in males as well as hermaphrodites; yet, disruption of a hermaphrodite-specific process is not expected to alter expression of the X chromosome in males. Paradoxically, of five X-linked genes assayed, *dpy-21* mutations caused an elevation in transcript levels from four (Meneely and Wood, 1987; Meyer and Casson, 1986) and a reduction in transcript levels from one (DeLong et al., 1987). Despite their altered transcript levels, *dpy-21* mutant XO males appear phenotypically wild type. Our results show that in males, the effect of *dpy-21* mutations on X-linked genes does not occur through the dosage compensation pathway. We stained XO male embryos with DPY-21 antibodies and an X-chromosome FISH probe to confirm that DPY-21 is expressed in males and to determine whether it localizes to X, as it does in hermaphrodites. In XO embryos of all ages, the pattern of localization resembled that in XX embryos that had not yet activated dosage compensation. DPY-21 was dispersed throughout the nucleus, in multiple foci of intense staining that were not coincident with the X chromosome (Fig. 4E). Thus, DPY-21 appears to influence gene expression in males through a route that is independent of dosage compensation. Consistent with this conclusion, mutations in the dosage compensation genes *dpy-26*, *dpy-27* or *dpy-28* do not suppress the reduction in X-linked gene expression caused by a *dpy-21* mutation (Plenefisch et al., 1989).

Activity of the dosage compensation proteins is switched off in males by the male-specific gene *xol-1*, which represses the hermaphrodite-specific *sdc* genes and thereby prevents recruitment of the dosage compensation complex to the male X chromosome (Davis and Meyer, 1997; Dawes et al., 1999; DeLong et al., 1993; Miller et al., 1988; Rhind et al., 1995). If DPY-21 is controlled by the genetic hierarchy that regulates other dosage compensation proteins, we would expect DPY-21

to be localized ectopically to the X chromosome of *xol-1* mutant XO animals. This expectation was met in the experiment of Fig. 4F.

Recruitment of DPY-21 to hermaphrodite X chromosomes requires all other members of the dosage compensation complex

In hermaphrodites, SDC-2, SDC-3 and DPY-30 are essential for the recruitment of all dosage compensation proteins to X chromosomes (Chuang et al., 1996; Lieb et al., 1998; Lieb et al., 1996). SDC-2 confers both hermaphrodite specificity and X-chromosome recognition to the dosage compensation process (Dawes et al., 1999). To explore the mechanism by which DPY-21 is recruited to X chromosomes, we determined whether mutation of individual dosage compensation genes blocks the recruitment of DPY-21 to X. In *sdc-2* and *sdc-3* mutants, DPY-21 was present but distributed throughout the nucleus in multiple foci of intense staining, as it is in males (Fig. 5A-C). These DPY-21 foci did not coincide with X chromosomes, as demonstrated by the combination of DPY-21 antibody staining and X-chromosome FISH (Fig. 5A-C). Therefore, SDC-2 and SDC-3 are required for the recruitment of DPY-21 to X.

In hermaphrodites, DPY-26, DPY-27, DPY-28 and MIX-1 have a complex dependence on each other for their stability and X localization. For example, without DPY-27, both DPY-26 and MIX-1 are stable but cannot localize to X (Lieb et al., 1998; Lieb et al., 1996). Without DPY-28, the DPY-26, DPY-27 and MIX-1 proteins are unstable (Chuang et al., 1996; Lieb et al., 1998; Lieb et al., 1996). Unlike these DPY proteins, DPY-21 does not require DPY-26, DPY-27 or DPY-28 for its accumulation and stability. However, DPY-21 does require DPY-26, DPY-27 and DPY-28 for its localization to X chromosomes (Fig. 5D-F). The dependence of DPY-21 on SDC and DPY proteins for its recruitment to X further indicates that DPY-21 downregulates X-linked gene expression in XX animals through the dosage compensation complex.

DPY-21 is not required for the stability or X localization of the other dosage compensation proteins, even though it is a member of the complex (Chuang et al., 1996; Davis and Meyer, 1997; Dawes et al., 1999; Lieb et al., 1998; Lieb et al., 1996). Our biochemial analysis further demonstrated this point. The level of MIX-1, a dual-functional protein with roles in both dosage compensation and chromosome condensation, is equivalent in wild-type and *dpy-21(428)* mutant extracts (Fig. 3A). Regarding the stability and localization of the complex, DPY-21 behaves like the dosage compensation complex member SDC-1 and unlike the other DPY proteins (Chuang et al., 1994; Davis and Meyer, 1997; Dawes et al., 1999; Lieb et al., 1998; Lieb et al., 1996). Nonetheless, the localization of DPY-21 and SDC-1 occurs independently. DPY-21 localizes to X chromosomes in *sdc-1* mutants (Fig. 5G), and SDC-1 localizes to X in *dpy-21* mutants (not shown).

DPY-21 participates directly in chromosome-wide repression but not in gene-specific repression

In addition to activating dosage compensation, SDC proteins induce hermaphrodite sexual differentiation by repressing transcription of the male-specific, autosomal gene *her-1* (Chu et al., 2002; Dawes et al., 1999; DeLong et al., 1993; Trent et al., 1991). SDC proteins repress *her-1* transcription 20-fold by binding to three separate regulatory regions within the gene (Chu et al., 2002). The first binding site overlaps the start point of *her-1* transcription. The second and third sites lie within the second intron of *her-1*; each contains a 15 bp sequence that is necessary for SDC localization. The SDC proteins recruit the DPY components of the X-chromosome dosage compensation complex to *her-1*, implying the direct participation of these DPY proteins in gene-specific repression (Chu et al., 2002). Given that DPY-21 is a member of the repression complex on X, we asked whether DPY-21 is a member of the repression complex on *her-1*.

Recruitment of proteins to *her-1* can be assayed in hermaphrodites carrying an extrachromosomal DNA array containing multiple tandem repeats of the *her-1* regulatory regions, *lac* operator repeats (Straight et al., 1996), and a transgene encoding LacI::GFP (Chu et al., 2002; Dawes et al., 1999). LacI::GFP binds to the *lac* operator sequence, allowing the array to be visualized by GFP fluorescence. If a protein is recruited to *her-1*, an antibody raised against the protein will co-localize with GFP fluorescence.

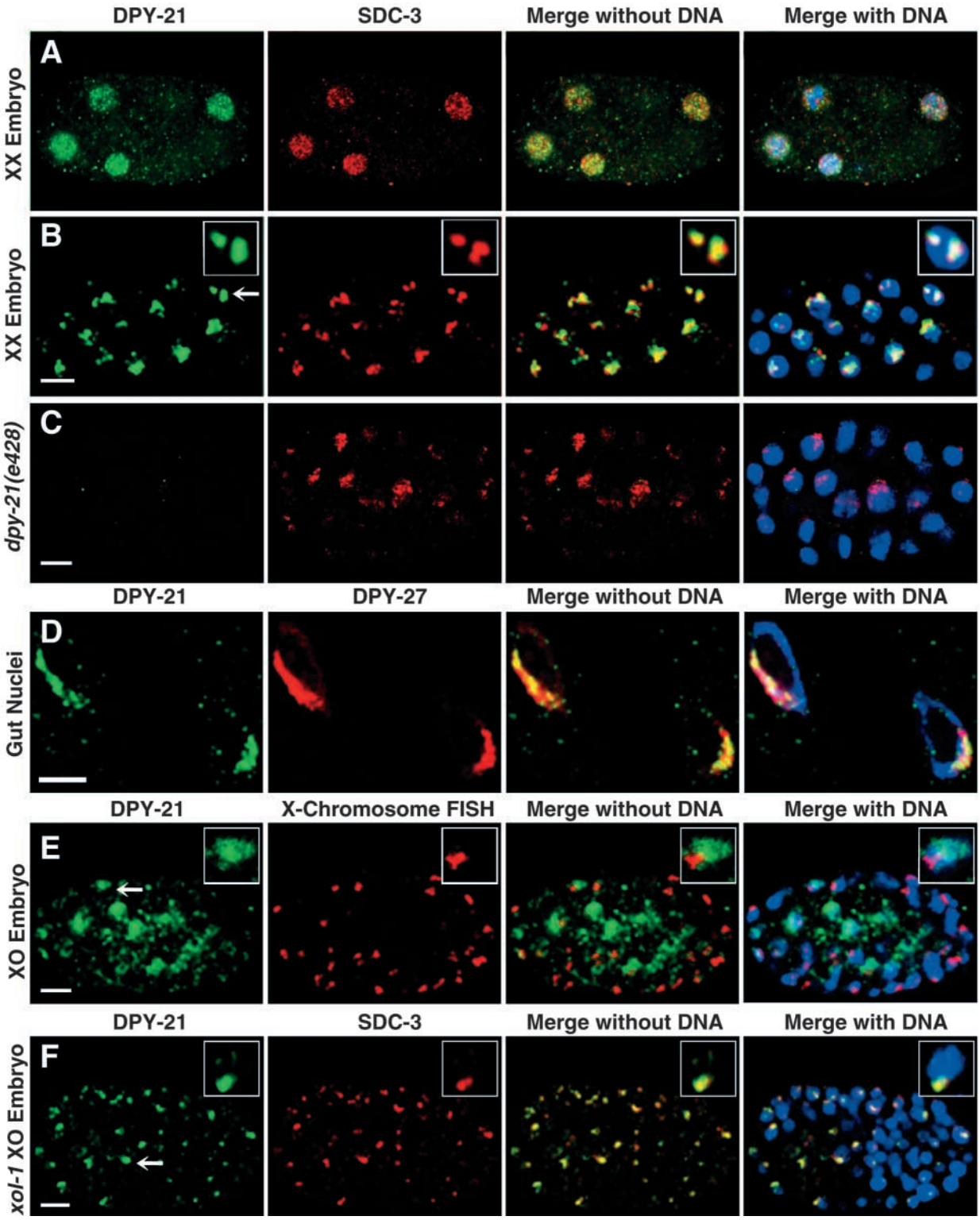
Surprisingly, DPY-21 was not recruited to *her-1*. In both embryos (Fig. 6A) and adult gut nuclei (Fig. 6B) co-stained with SDC-3 and DPY-21 antibodies, SDC-3 co-localized with *her-1* arrays and X chromosomes, but DPY-21 only co-localized with X chromosomes. DPY-21 provides the first indication that the repression complex on X differs from the repression complex on *her-1*.

SDC-3 is pivotal in *her-1* recognition while SDC-2 is pivotal in X-chromosome recognition

Having found a difference in the composition of repression complexes at *her-1* versus X, we determined whether the two repression complexes recognize their DNA targets in a similar or different way. SDC-2 confers X-chromosome recognition. It can localize to X chromosomes in the absence of other components of the dosage compensation complex, and it triggers assembly of these components onto X chromosomes (Dawes et al., 1999). We addressed whether SDC-2 was equivalently important for *her-1* recognition. We found that SDC-3 played the more central role in recognizing *her-1*.

While examining the localization of SDC-3 in young embryos (<30 cells) we noticed that SDC-3 could associate with *her-1* regulatory regions at a time well before it localized to X chromosomes (Fig. 7A,B), suggesting another difference between the X and *her-1* repression complexes. To verify this result, we assayed SDC-3 localization in four strains (Table 1A). Two strains contained independent arrays with all three SDC binding sites in *her-1*, and a third strain contained arrays with only SDC binding sites 2 and 3 (Fig. 7G). The fourth strain contained control arrays with only the vector sequences present in the other arrays. In embryos with fewer than 20 cells, SDC-3 localized infrequently to X chromosomes or to arrays with vector sequence only, but localized robustly to the arrays carrying *her-1* regulatory regions (Table 1A). Thus, SDC-3 localizes to *her-1* earlier than it localizes to X.

The fact that SDC-3 localized to *her-1* before SDC-2 was detectable in embryos suggested that recruitment of SDC-3 to *her-1* was independent of SDC-2. To test whether SDC-3 localization to *her-1* is indeed SDC-2 independent, we examined SDC-3 localization in *sdc-2*(null) mutant embryos carrying *her-1* arrays. SDC-3 co-localized with *her-1* arrays



but not with X chromosomes in *sdc-2*(null) mutants (Table 1B, Fig. 7C,D), demonstrating that localization of SDC-3 to *her-1* does not require SDC-2. By contrast, SDC-2 failed to co-localize with *her-1* arrays in *sdc-3*(null) mutants (Fig. 7F). Thus, although SDC-2 can localize to X without other dosage compensation proteins, SDC-2 requires SDC-3 for its recruitment to *her-1*. Conversely, SDC-3 localizes to *her-1* independently of SDC-2, but requires SDC-2 for its localization to X. SDC-3 thus appears to play the lead role in recognizing *her-1* sequences, whereas SDC-2 plays the lead role in recognizing X sequences.

The dependence of SDC-3 on other dosage compensation proteins for its recruitment to *her-1* was also examined. SDC-3 is expressed in low levels in *dpy-27*(null) mutants and does

Fig. 4. DPY-21 localizes to the X chromosomes of XX but not XO embryos, as expected for a component of the dosage compensation complex. (A-F) False-color confocal images of wild-type XX embryos (A,B), *dpy-21(e428)* mutant XX embryos (C), wild-type hermaphrodite gut nuclei (D), *him-8(e1489)* XO embryos (E) and *him-8(e1489); xol-1(y9)* mutant XO embryos (F) stained with DPY-21 antibodies (green), the DNA-intercalating dye 4′,6 diamidino-2-phenylindole (DAPI) (blue) and an X-chromosome-specific marker (red) (either an X-chromosome-specific FISH probe or antibodies to SDC-3 or GFP, which identifies the DPY-27::GFP fusion protein used in D). (A-C,E,F) DPY-21 antibodies to amino acids 467-1102; (D) antibodies to the DPY-21 N terminus. The third column shows a merged image of the first two columns, and yellow indicates overlap in staining of DPY-21 and the X-chromosome marker. The fourth column shows the superimposition of DAPI images with images from the first two columns. Insets show the enlargement of a single nucleus indicated by the arrow. (A) In young embryos (<40 cells) that have not yet recruited the dosage compensation complex to X, DPY-21 is distributed throughout the nuclei. (B) In 40-cell stage embryos, DPY-21 exhibits a punctate pattern that is coincident with the X-localized SDC-3 protein. (C) Specificity of the DPY-21 antibody was shown in part by the absence of DPY-21 staining in *dpy-21(e428)* and *dpy-21(y59)* (data not shown), both of which contain an early amber stop mutation. SDC-3 localized to the X chromosomes of a *dpy-21* mutant, indicating that DPY-21 is not essential for the recruitment of the dosage compensation complex to X. (D) The X-chromosome localization of DPY-21 is maintained throughout hermaphrodite development, as shown by the X-chromosome localization of DPY-21 in adult gut nuclei, which carry a DPY-27::GFP fusion protein. (E) DPY-21 is expressed, but fails to localize to the X chromosome of XO animals. (F) In *xol-1(y9)* mutant XO embryos, which have inappropriately activated dosage compensation, both DPY-21 and SDC-3 co-localize with the single X chromosome, indicating that the X-chromosome localization of DPY-21 is under the same sex-specific control as SDC-3. Scale bars: 5 μm.

not localize to X chromosomes (Davis and Meyer, 1997). By contrast, we found that SDC-3, like SDC-2 (Chu et al., 2002), localizes to *her-1* arrays in *dpy-27*(null) mutant embryos (Fig. 7E, Table 1B), providing another example that SDC-3 has different requirements for its recruitment to *her-1* versus X. Together these results demonstrate that SDC-2 and SDC-3 assemble onto their regulatory targets in a different order and have different requirements for their localization. Moreover, different SDC proteins are pivotal for the recognition of *her-1* versus X-chromosome targets.

Discussion

A direct role for DPY-21 in dosage compensation

The molecular analysis presented here revealed the direct participation of DPY-21 in the dosage compensation process. DPY-21 associates physically with other components of the dosage compensation complex and localizes to X chromosomes of XX embryos to repress gene expression. Recruitment of DPY-21 to X chromosomes is regulated by the genetic hierarchy that coordinately controls sex determination and dosage compensation. The discovery of DPY-21 as a member of the X-chromosome dosage compensation complex was somewhat unexpected given the relative weakness of *dpy-21* mutant phenotypes compared with those caused by the loss of other members. Unlike other dosage compensation mutations, *dpy-21* mutations do not cause extensive XX-specific lethality, nor do they overtly disrupt the stability or localization of the dosage compensation complex. All 24 *dpy-21* alleles cause very similar phenotypes: elevated X-linked gene expression and an XX-specific dumpy, egg-laying-defective phenotype, both characteristic of rare adults that survive the lethality of more severe *dpy* mutations. The strongest *dpy-21* alleles cause XX-specific lethality, but only at a low level (17%). Our molecular and biochemical analysis indicates that the phenotype of the strongest *dpy-21* mutations represents the null phenotype. Previous genetic tests on this point had been inconclusive because the chromosomal deletions that remove *dpy-21* are haploinsufficient on their own.

Table 1. Localization of SDC-3 to *her-1* arrays

Genotype (array)	% SDC-3 localization*	Number of arrays with SDC-3 localization	Total number of arrays
A			
Wild type (no *her-1* binding sites, *yEx300*)	17.6	34	193
Wild type (3 *her-1* binding sites, *yEx658*)	42.2	97	230
Wild type (3 *her-1* binding sites, *yEx374*)	50.7	151	298
Wild type (*her-1* binding sites 2, 3, *yEx333*)	88.4	190	215
B			
xol-1 (no *her-1* binding sites, *yEx676*)	10.2	60	591
xol-1 (3 *her-1* binding sites, *yEx576*)	93.9	641	683
dpy-27(y167); *xol-1* (3 *her-1* binding sites, *yEx501*)	49.5	299	604
xol-1 sdc-2(y74) (3 *her-1* binding sites, *yEx502*)	53.2	402	756

SDC-3 localizes to *her-1* arrays before it is recruited to X chromosomes. (A) SDC-3 localizes to *her-1* arrays in young embryos before the dosage compensation complex is recruited to X chromosomes. In each of several embryos with fewer than 20 cells, five *her-1* arrays were randomly chosen and assayed for SDC-3 localization. (B) Recruitment of SDC-3 to *her-1* arrays is not dependent on SDC-2 or DPY-27, unlike its recruitment to X. A summary is provided for the quantification of SDC-3 localization to *her-1* arrays in embryos from the following strains: (1) *xol-1(y9) X; yEx676*; (2) *him-5(e1490) V; xol-1(y9) X; yEx576*; (3) *unc-32(e189) dpy-27(y167) III; flu-2(e1003) xol-1(y9) X; yEx501*; and (4) *her-1(hv1y101) V; xol-1(y9) sdc-2(y74) unc-9(e101) X; yEx502*. These last two strains were maintained as XO hermaphrodites.

*SDC-3 localization to *her-1* arrays was significantly different from its localization to arrays without *her-1*, as determined by Fisher Exact test to $P<0.001$. In all previous array experiments conducted with different antibodies and promoters in unrelated studies, a 10-20% background level of co-localization is normal and therefore considered insignificant (Chu et al., 2002; Dawes et al., 1999).

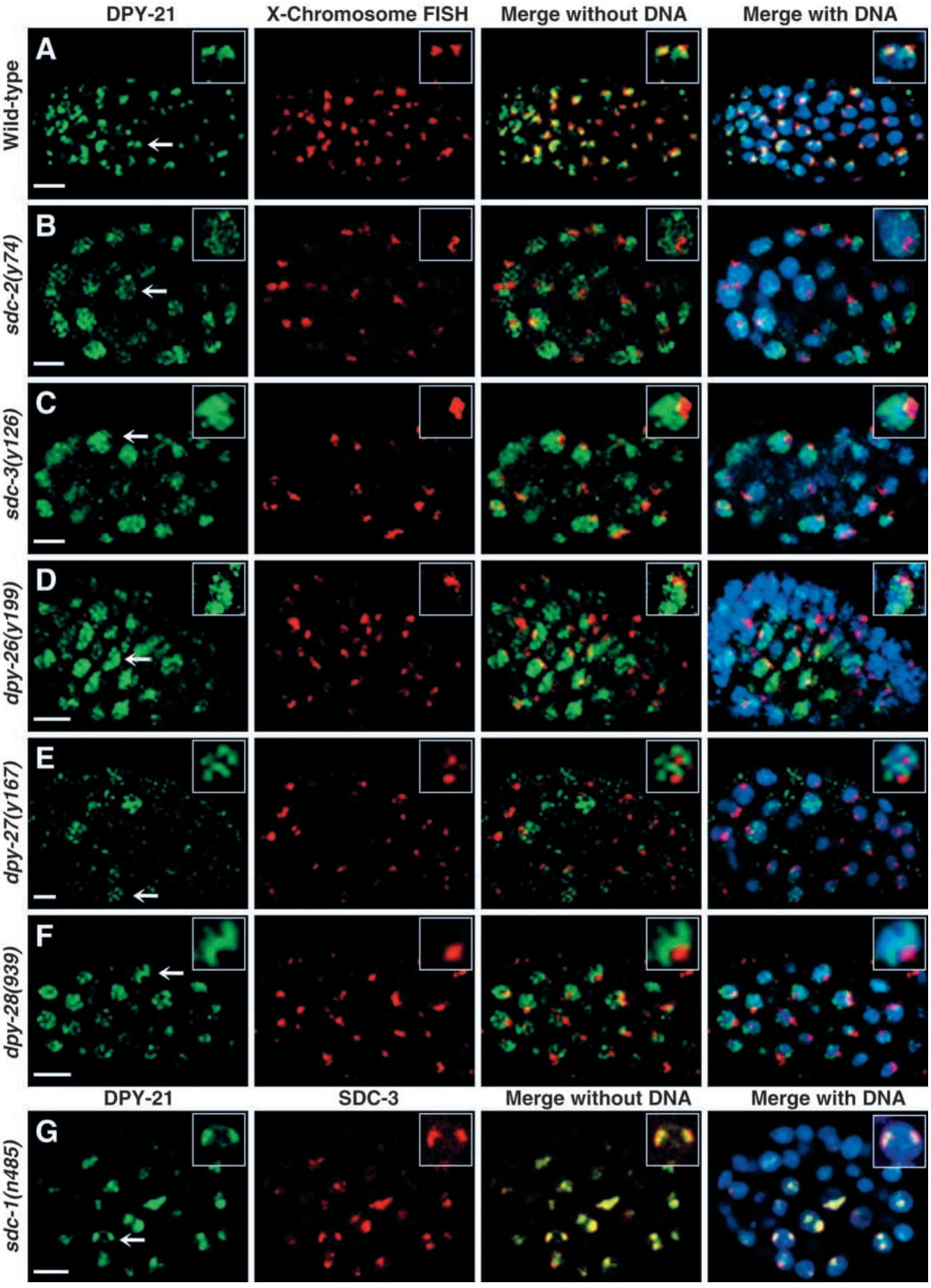

DPY-21
X-Chromosome FISH
Merge without DNA
Merge with DNA
A
Wild-type
B
sdc-2(y74)
C
sdc-3(y126)
D
dpy-26(y199)
E
dpy-27(y167)
F
dpy-28(939)
DPY-21
SDC-3
Merge without DNA
Merge with DNA
G
sdc-1(n485)

Fig. 5. DPY-21 is recruited to X chromosomes by components of the dosage compensation complex. (A-G) Confocal images of wild-type (A), *sdc-2(y74)* (B), *sdc-3(y126)* (C), *dpy-26(y199)* (D), *dpy-27(y167)* (E), *dpy-28(s939)* (F) and *sdc-1(n485)* (G) mutant embryos co-stained with DPY-21 antibodies to amino acids 467-1102 (green), DAPI (blue) and an X-chromosome-specific FISH probe (red) or SDC-3 antibodies (red). (A) In wild-type embryos, foci of DPY-21 staining co-localize with X chromosomes identified by FISH. (B-F) DPY-21 accumulates in dosage compensation mutant embryos, but foci of DPY-21 staining in *sdc-2(y74)* (B), *sdc-3(y126)* (C), *dpy-26(y199)* (D), *dpy-27(y167)* (E) and *dpy-28(s939)* (F) mutants are not coincident with the X chromosome. Thus DPY-21 requires *sdc-2*, *sdc-3*, *dpy-26*, *dpy-27* and *dpy-28* for its localization to X but not for its stability. (G) By contrast, neither DPY-21 nor SDC-3 requires *sdc-1* for its localization to X. Insets show the enlargement of a single nucleus indicated by the arrow. Scale bars: 5 μm.

The partial disruption of dosage compensation caused by *dpy-21* null mutations can now be understood in the context of DPY-21 behavior. Although DPY-21 is a member of the dosage compensation complex, its association with the complex is not as stable as that of other members. Antibodies to dosage compensation proteins do not immunoprecipitate DPY-21 as readily as other components. Moreover, the stability of DPY-21 does not depend on the presence of other dosage compensation proteins, further suggesting that DPY-21 is not as integral to the complex as other members. Finally, recruitment of DPY-21 to X requires all other dosage compensation proteins except SDC-1. By contrast, the stability and/or X localization of these other dosage compensation proteins requires only a subset of dosage compensation proteins, implying an earlier and/or stronger association with the complex than DPY-21.

How might DPY-21 regulate X-chromosome gene expression? The DPY-21 protein sequence provided no clue as to its function. DPY-21, unlike the dosage compensation proteins DPY-26, DPY-27 and MIX-1, has no similarity to subunits of condensin, a complex that controls mitotic and meiotic chromosome structure. Rather than functioning in X-chromosome gene regulation through modification of X-chromosome structure, as proposed for the other proteins, DPY-21 might modify the activity of the dosage compensation complex, for example through a covalent modification or an allosteric effect. A more intriguing possibility is that DPY-21 might act directly on X chromatin to stabilize a repressed chromatin state initiated by the core dosage compensation complex, perhaps through histone modification or a direct association with chromatin.

Targeting of SDC proteins to *her-1* versus X-chromosome regulatory regions

The dosage compensation complex associates with X chromosomes of hermaphrodites to repress transcription twofold, while it associates with the regulatory sites of the male sex-determination gene *her-1* to repress transcription 20-fold (Chu et al., 2002; Dawes et al., 1999). Our experiments revealed the first molecular differences in the repression at *her-1* versus X: in the composition and recruitment of repression complexes and in the proteins that recognize these DNA targets.

Although DPY-21 localizes to X chromosomes as a component of the dosage compensation complex, it does not localize to *her-1*, unlike the other dosage compensation proteins. It is not yet known how this difference in DPY-21 localization contributes to the mechanisms of gene-specific versus chromosome-wide repression. DPY-21 does not, for

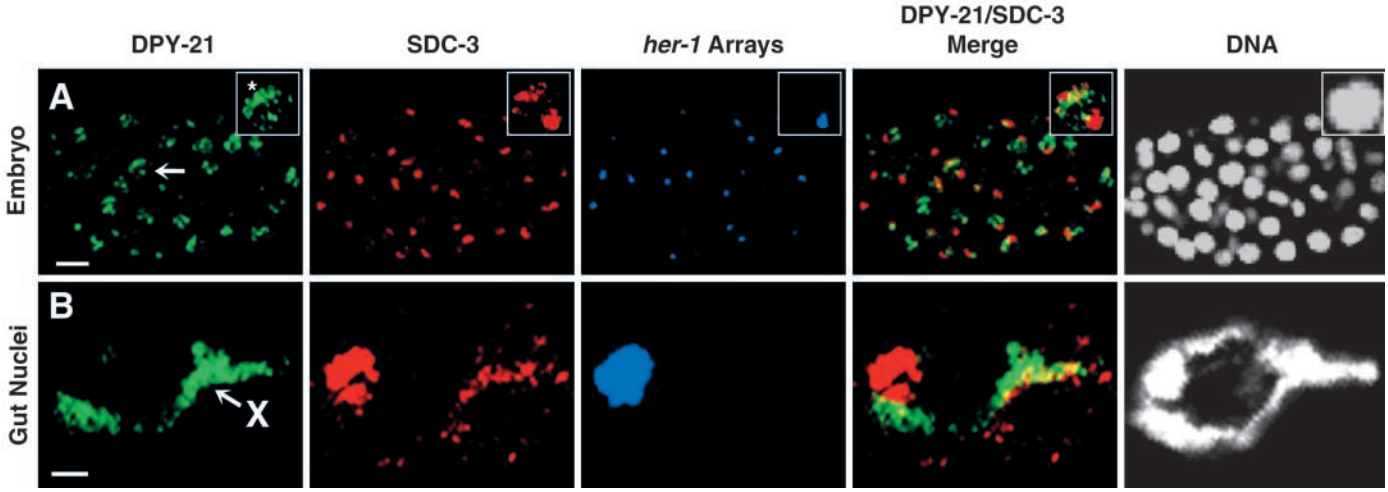


Fig. 6. DPY-21 is not recruited to *her-1* regulatory regions in XX animals, unlike other components of the dosage compensation complex. (A,B) False-color confocal immunofluorescence images of wild-type embryos (A) or gut cell nuclei (B) carrying *her-1* extrachromosomal arrays that contain multiple copies of *her-1* regulatory regions, *lac* operator repeats (*lacO*) and a transgene encoding a LacI-GFP fusion protein. LacI-GFP repressor binding to *lacO* permits array detection by GFP antibodies. For each embryo or gut cell nucleus, a single *z*-section is shown. Embryos and gut cell nuclei were stained with DAPI (gray) and antibodies to DPY-21 (green), SDC-3 (red) and GFP (blue). The fourth column shows the superimposition of the DPY-21 and SDC-3 images. (A) In embryos that have activated dosage compensation, both DPY-21 and SDC-3 co-localize with the X chromosome, which is denoted by an asterisk in the inset. By contrast, SDC-3, but not DPY-21, localizes to *her-1* regulatory regions on the arrays. (B) In adult gut cell nuclei, DPY-21 co-localizes with SDC-3 on X chromosomes, but does not co-localize with SDC-3 on *her-1* regulatory regions. Thus, DPY-21 participates directly in the chromosome-wide repression of X but not in the gene-specific repression of *her-1*. Scale bars: 5 μm.

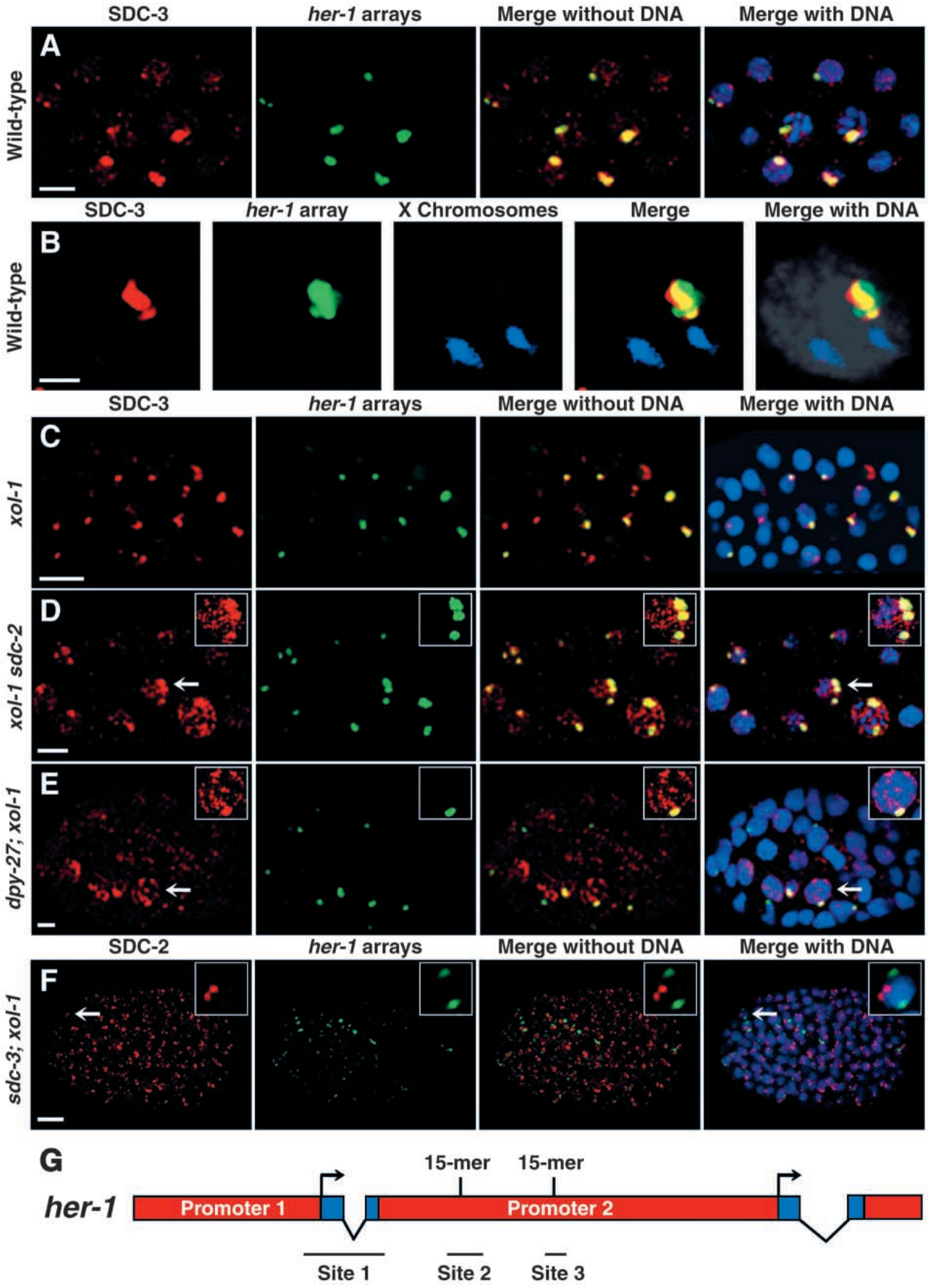

SDC-3
her-1 arrays
Merge without DNA
Merge with DNA
A
Wild-type
SDC-3
her-1 array
X Chromosomes
Merge
Merge with DNA
B
Wild-type
SDC-3
her-1 arrays
Merge without DNA
Merge with DNA
C
xol-1
D
xol-1 sdc-2
E
dpy-27; xol-1
SDC-2
her-1 arrays
Merge without DNA
Merge with DNA
F
sdc-3; xol-1
G
her-1
15-mer
15-mer
Promoter 1
Promoter 2
Site 1
Site 2
Site 3

Fig. 7. SDC-3 localization to *her-1* regulatory regions does not require DPY-27 or SDC-2, unlike its localization to X chromosomes. (A-E) False-color confocal immunofluorescence images of <30-cell wild-type embryos bearing extrachromosomal arrays carrying multiple copies of *her-1* regulatory sites 2 and 3 (A,B), or dosage compensation mutant embryos carrying *her-1* regulatory sites 1, 2 and 3 (C-E) plus *lacO* repeats and a transgene encoding LacI-GFP. Embryos were stained with DAPI (blue) and antibodies to SDC-3 (red) and GFP (green) (A,C-E). (A) In embryos with less than 30 cells, SDC-2 is not detectable (data not shown) and SDC-3 co-localizes with *her-1* regulatory regions. (B) A single nucleus from an embryo stained with SDC-3 antibodies (red) and FISH probes specific to *her-1* arrays (green) and X chromosomes (blue). Overlapping patterns of SDC-3 and *her-1* array staining are indicated by yellow. In this single nucleus of a 10-cell embryo, SDC-3 localizes to *her-1* regulatory regions at a time prior to its recruitment to X chromosomes. These results suggest that SDC-3 can localize to *her-1* independently of SDC-2. (C) In *xol-1* mutant embryos, *her-1* transcription is repressed by the SDC proteins and SDC-3 was found localized to *her-1* regulatory regions. (D) SDC-3 localizes to *her-1* regulatory regions in *xol-1 sdc-2* mutant embryos, indicating that SDC-3 does not require SDC-2 for its localization to *her-1*. By contrast, SDC-3 requires SDC-2 for its recruitment to X. The lack of SDC-2 protein was confirmed by staining with anti-SDC-2 antibodies (data not shown). (E) In *dpy-27; xol-1* mutant embryos, SDC-3 is recruited to *her-1* arrays, albeit in a mosaic pattern. (F) False-color confocal immunofluorescence images of an older *sdc-3; xol-1* mutant embryo carrying extrachromosomal arrays of *her-1* regulatory sequences and stained with SDC-2 antibodies. This image shows that SDC-2 requires SDC-3 for its localization to *her-1* but its X localization is not perturbed by loss of SDC-3. Together, these results indicate that SDC-3 can bind to *her-1* regulatory regions independently of DPY-27 and SDC-2. Moreover, the SDC protein required for recognition of *her-1* regulatory sequences differs from that required for X-chromosome recognition. (G) Schematic of the *her-1* gene and the binding sites for the dosage compensation complex. Transcription from the P1 promoter produces the functional male-specific *her-1* transcript (1.2 kb) that includes four exons (blue). A second promoter resides within the second intron of *her-1*. This second promoter is co-regulated with the first promoter and makes a 0.8 kb transcript of unknown function that includes the last two exons. Insets show the enlargement of a single nucleus indicated by the arrow. Scale bars: 1 μm for B; 5 μm for A,C-F.

example, counteract the activity of other dosage compensation proteins to limit the degree of X repression to only twofold. If it did, *dpy-21* mutations would cause enhanced X repression rather than the diminished repression observed. Perhaps DPY-21 is absent from *her-1* because its replacement in the repression complex with a different protein would increase the repressive capacity of the complex. Alternatively, DPY-21 may have a mechanism of action on X that would be detrimental to the development or viability of the organism if DPY-21 were allowed to bind to *her-1*. For example, if DPY-21 helps transmit the repressed chromatin state along X, then binding of DPY-21 to *her-1* might adversely influence the activity of genes neighboring *her-1*.

We have shown that SDC-2 and SDC-3 have different protein requirements for recruitment to their targets at *her-1* versus X. Moreover, the SDC protein sufficient for *her-1* target recognition is different from the one sufficient for X target recognition. SDC-2 can localize to X independently of SDC-3, but requires SDC-3 for its localization to *her-1*. SDC-2 triggers assembly of dosage compensation proteins onto X chromosomes and appears pivotal for X-chromosome recognition (Dawes et al., 1999). By contrast, SDC-3 can localize to *her-1* independently of SDC-2 but requires SDC-2 for its localization to X. Similarly, SDC-3 does not require DPY-27 for its association with *her-1* but it does require DPY-27 for its association with X. SDC-3 is essential for the localization of all dosage compensation proteins to *her-1*, and SDC-3 appears pivotal for *her-1* target recognition.

These results concur with and extend previous genetic analysis indicating that SDC-3 functions differently at *her-1* versus X. More specifically, the sex determination and dosage compensation activities of SDC-3, unlike SDC-2, are separately mutable: SDC-3 requires its zinc fingers to localize to X but its ATP binding motif to localize to *her-1* (Chu et al., 2002; Dawes et al., 1999; DeLong et al., 1993; Klein and Meyer, 1993). The initial mapping of recognition elements within *her-1* identified three SDC binding sites, each of which differed in sequence, but two of which shared an identical 15 bp sequence essential for SDC binding (Chu et al., 2002). Because that 15 base pair sequence is not present on X chromosomes, it cannot be central for the recruitment of dosage compensation components to X chromosomes. This observation can now be interpreted in light of our finding that different SDC proteins play lead roles in recognizing *her-1* versus X regulatory targets. The 15 bp sequence might be part of a recognition sequence used by SDC-3 but not by SDC-2. In conclusion, our analysis has revealed important molecular differences in the composition and targeting of protein complexes that achieve both gene-specific and chromosome-wide regulation, opening the way to a deeper understanding of the mechanisms underlying these two major forms of gene regulation.

We thank L. DeLong for genetic analysis of the *dpy-21* mutations; E. Cookson for initial SNP mapping of *dpy-21*; R. Chan, D. Chu, G. Csankovszki, E. Ralston, D. Reiner and C. Tsai for reagents and advice on experimental design; and R. Chan, T. Cline, E. Ralston, A. Severson and C. Tsai for thoughtful comments on the manuscript. This work was funded in part by NIH Grant R37 GM30702 to B.J.M. and NIH pre-doctoral training grants GM07232 and HD07375 to S.A.Y. B.J.M. is an investigator of the Howard Hughes Medical Institute.

References

Chu, D. S., Dawes, H. E., Lieb, J. D., Chan, R. C., Kuo, A. F. and Meyer, B. J. (2002). A molecular link between gene-specific and chromosome-wide transcriptional repression. *Genes Dev.* **16**, 796-805.

Chuang, P.-T., Albertson, D. G. and Meyer, B. J. (1994). DPY-27: a chromosome condensation protein homolog that regulates *C. elegans* dosage compensation through association with the X chromosome. *Cell* **79**, 459-474.

Chuang, P.-T., Lieb, J. D. and Meyer, B. J. (1996). Sex-specific assembly of a dosage compensation complex on the nematode X chromosome. *Science* **274**, 1736-1739.

Davis, T. L. and Meyer, B. J. (1997). SDC-3 coordinates the assembly of a dosage compensation complex on the nematode X chromosome. *Development* **124**, 1019-1031.

Dawes, H. E., Berlin, D. S., Lapidus, D. M., Nusbaum, C., Davis, T. L. and Meyer, B. J. (1999). Dosage compensation proteins targeted to X chromosomes by a determinant of hermaphrodite fate. *Science* **284**, 1800-1804.

DeLong, L., Casson, L. P. and Meyer, B. J. (1987). Assessment of X chromosome dosage compensation in *Caenorhabditis elegans* by phenotypic analysis of *lin-14*. *Genetics* **117**, 657-670.

DeLong, L. D., Plenefisch, J. D., Klein, R. D. and Meyer, B. J. (1993). Feedback control of sex determination by dosage compensation revealed through *Caenorhabditis elegans sdc-3* mutations. *Genetics* **133**, 875-896.

Dernburg, A. F. and Sedat, J. W. (1998). Mapping three-dimensional chromosome architecture in situ. *Methods Cell Biol.* **53**, 187-233.

Hagstrom, K. A., Holmes, V. F., Cozzarelli, N. R. and Meyer, B. J. (2002). *C. elegans* condensin promotes mitotic chromosome architecture, centromere organization, and sister chromatid segregation during mitosis and meiosis. *Genes Dev.* **16**, 729-742.

Hodgkin, J. (1983). *X* chromosome dosage and gene expression in *Caenorhabditis elegans*: two unusual dumpy genes. *Mol. Gen. Genet.* **192**, 452-458.

Howe, M., McDonald, K. L., Albertson, D. G. and Meyer, B. J. (2001). HIM-10 is required for kinetochore structure and function on *Caenorhabditis elegans* holocentric chromosomes. *J. Cell Biol.* **153**, 1227-1238.

Klein, R. D. and Meyer, B. J. (1993). Independent domains of the *sdc-3* protein control sex determination and dosage compensation in *C. elegans*. *Cell* **72**, 349-364.

Lieb, J. D., Capowski, E. E., Meneely, P. and Meyer, B. J. (1996). DPY-26, a link between dosage compensation and meiotic chromosome segregation in the nematode. *Science* **274**, 1732-1736.

Lieb, J. D., Albrecht, M. R., Chuang, P.-T. and Meyer, B. J. (1998). MIX-1: An essential component of the *C. elegans* mitotic machinery executes X chromosome dosage compensation. *Cell* **92**, 265-277.

Meller, V. H. and Kuroda, M. I. (2002). Sex and the single chromosome. *Adv. Genet.* **46**, 1-24.

Meneely, P. M. and Wood, W. B. (1984). An autosomal gene that affects X chromosome expression and sex determination in Caenorhabditis elegans. *Genetics* **106**, 29-44.

Meneely, P. M. and Wood, W. B. (1987). Genetic analysis of *X*-chromosome dosage compensation in *Caenorhabditis elegans*. *Genetics* **117**, 25-41.

Meyer, B. J. (2000). Sex in the worm: counting and compensating X-chromosome dose. *Trends Genet.* **16**, 247-253.

Meyer, B. J. and Casson, L. P. (1986). *Caenorhabditis elegans* compensates for the difference in X chromosome dosage between the sexes by regulating transcript levels. *Cell* **47**, 871-881.

Miller, L. M., Plenefisch, J. D., Casson, L. P. and Meyer, B. J. (1988). *xol-1*: a gene that controls the male modes of both sex determination and X chromosome dosage compensation in *C. elegans*. *Cell* **55**, 167-183.

Nusbaum, C. and Meyer, B. J. (1989). The *Caenorhabditis elegans* gene *sdc-2* controls sex determination and dosage compensation in XX animals. *Genetics* **122**, 579-593.

Plenefisch, J. D., DeLong, L. and Meyer, B. J. (1989). Genes that implement the hermaphrodite mode of dosage compensation in *Caenorhabditis elegans*. *Genetics* **121**, 57-76.

Rhind, N. R., Miller, L. M., Kopczynski, J. B. and Meyer, B. J. (1995). *xol-1* acts as an early switch in the *C. elegans* male/hermaphrodite decision. *Cell* **80**, 71-82.

Straight, A., Belmont, A., Robinett, C. and Murray, A. (1996). GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr. Biol.* **6**, 1599-1608.

Trent, C., Purnell, B., Gavinski, S., Hageman, J. and Wood, W. B. (1991). Sex-specific transcriptional regulation of the *C. elegans* sex-determining gene *her-1*. *Mech. Dev.* **34**, 43-56.

Villeneuve, A. M. and Meyer, B. J. (1987). *sdc-1*: a link between sex determination and dosage compensation in *C. elegans*. *Cell* **48**, 25-37.

Wutz, A. and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* **5**, 695-705.