

A molecular link between gene-specific and chromosome-wide transcriptional repression

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Gene-specific and chromosome-wide mechanisms of transcriptional regulation control development in multicellular organisms. SDC-2, the determinant of hermaphrodite fate in *Caenorhabditis elegans*, is a paradigm for both modes of regulation. SDC-2 represses transcription of X chromosomes to achieve dosage compensation, and it also represses the male sex-determination gene *her-1* to elicit hermaphrodite differentiation. We show here that SDC-2 recruits the entire dosage compensation complex to *her-1*, directing this X-chromosome repression machinery to silence an individual, autosomal gene. Functional dissection of *her-1* in vivo revealed DNA recognition elements required for SDC-2 binding, recruitment of the dosage compensation complex, and transcriptional repression. Elements within *her-1* differed in location, sequence, and strength of repression, implying that the dosage compensation complex may regulate transcription along the X chromosome using diverse recognition elements that play distinct roles in repression.

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The complex patterns of gene expression that control development are established by multiple regulatory mechanisms that operate locally on individual genes or globally across entire chromosomes or subchromosomal domains. These gene-specific and chromosome-wide modes of regulation are generally controlled by different protein complexes; however, a small number of global repressors have also been shown to function as local silencers of individual genes (Dawes et al. 1999; Moazed 2001; Nielsen et al. 2001). For example, the Sir proteins of *Saccharomyces cerevisiae* repress transcription at individual silent mating-type loci as well as telomeric regions of chromosomes (Aparicio et al. 1991; Moretti et al. 1994). The SDC-2 (sex-determination and dosage compensation) chromatin-binding protein of *Caenorhabditis elegans* has the versatility to repress transcription of an autosomal sex-determination gene by 20-fold and the entire X chromosome by twofold (Nusbaum and Meyer 1989; Trent et al. 1991; Dawes et al. 1999; Meyer 2000). Such dual-function regulatory components provide unique opportunities to explore mechanisms of local and global regulation by analyzing

the more tractable mechanisms of gene-specific regulation.

SDC-2 coordinates all hermaphrodite-specific aspects of *C. elegans* development (Nusbaum and Meyer 1989). Acting as a gene-specific repressor, SDC-2 induces hermaphrodite sexual differentiation in XX animals by repressing transcription of the male (XO) sex-determining gene *her-1* (Trent et al. 1991; Dawes et al. 1999). Acting simultaneously as a chromosome-wide repressor, SDC-2 activates dosage compensation by triggering the assembly of a specialized protein complex onto hermaphrodite X chromosomes to reduce X-linked gene expression by half (Dawes et al. 1999). The dosage compensation complex (Chuang et al. 1996; Lieb et al. 1996, 1998) resembles the condensin complex, which drives mitotic chromosome compaction in vitro (Koshland and Strunnikov 1996; Hirano 2000). X-chromosome repression is essential and equalizes gene expression between the sexes. Failure to dosage compensate causes hermaphrodite lethality.

Here we show that SDC-2 recruits all known components of the X-chromosome dosage compensation complex to the autosomal *her-1* gene, allowing us to perform a detailed dissection in vivo of DNA recognition elements essential for SDC binding, recruitment of the dosage compensation complex, and transcriptional regulation. Within *her-1*, diverse DNA elements specify SDC binding and distinct levels of repression. Thus, a chromosome-wide repression complex can achieve different degrees of repression by associating with diverse DNA targets.

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Results

SDC proteins function as a complex in vivo to repress her-1 and X chromosomes

How does SDC-2 discriminate between different targets and repress them to different degrees? To answer this question, we first identified proteins that function with SDC-2 at *her-1*. *sdc-2* was known to interact genetically with *sdc-1* and *sdc-3* to implement sex determination and dosage compensation in XX animals (Villeneuve and Meyer 1990; Davis and Meyer 1997; Dawes et al. 1999), but the precise molecular roles of SDC-1 and SDC-3 were not understood. Here we present three lines of evidence that the three SDC proteins form a complex in vivo to repress *her-1* and X chromosomes directly.

First, SDC-1, SDC-2, and SDC-3 all colocalize to X chromosomes and to *her-1* regulatory regions in vivo. Hermaphrodites carrying multiple tandem copies of *her-1* regulatory regions on GFP-tagged extrachromosomal arrays were stained with affinity-purified SDC antibodies (see Materials and Methods). SDC protein localization was assessed in adult intestinal nuclei, whose large size and polyploid DNA content facilitate the assay. The highly charged SDC-2 protein, which bears a coiled-coil motif, localized to X chromosomes and *her-1* arrays in adult gut cells (Fig. 1A), as shown previously in embryos (Dawes et al. 1999), thus validating the assay. The zinc-finger proteins SDC-1 and SDC-3 (Nonet and Meyer 1991; Klein and Meyer 1993) colocalized with SDC-2 at *her-1* and on X chromosomes (Fig. 1A,B), consistent with a direct role for these proteins in *her-1* repression and dosage compensation. In XX animals carrying an *sdc-3*(Tra) mutation, localization of SDC-1, SDC-2, and SDC-3 to *her-1* was greatly reduced, but localization to the X chromosome appeared unaffected (Fig. 1F; data not shown), consistent with the mutation impairing sex determination but not dosage compensation (DeLong et al. 1993). *sdc-3*(Tra) derepresses *her-1* transcription, causing 100% of XX animals to be severely masculinized by disrupting a putative ATP-binding motif in SDC-3 (DeLong et al. 1993; Klein and Meyer 1993). Therefore, SDC-1, SDC-2, and SDC-3 are localized appropriately to achieve both gene-specific and chromosome-wide repression.

Second, the SDC proteins interact physically to form a complex. Antibodies to any one of the SDC proteins co-immunoprecipitated all three SDC proteins from wild-type embryonic extracts (Fig. 2B). These precipitation reactions were specific, because none of the preimmune sera precipitated any of the SDC proteins (Fig. 2B), and none of the SDC antibodies precipitated (data not shown) or identified (Fig. 2A) their cognate proteins from extracts of the respective *sdc* null mutants.

Third, the SDC complex represses transcription of *her-1*, since ectopic production of SDC proteins in XO animals (normally males) induced hermaphrodite sexual development. SDC-2 is normally expressed only in XX animals, and ectopic expression of SDC-2 transformed 36% of XO animals into hermaphrodites (Dawes et al.

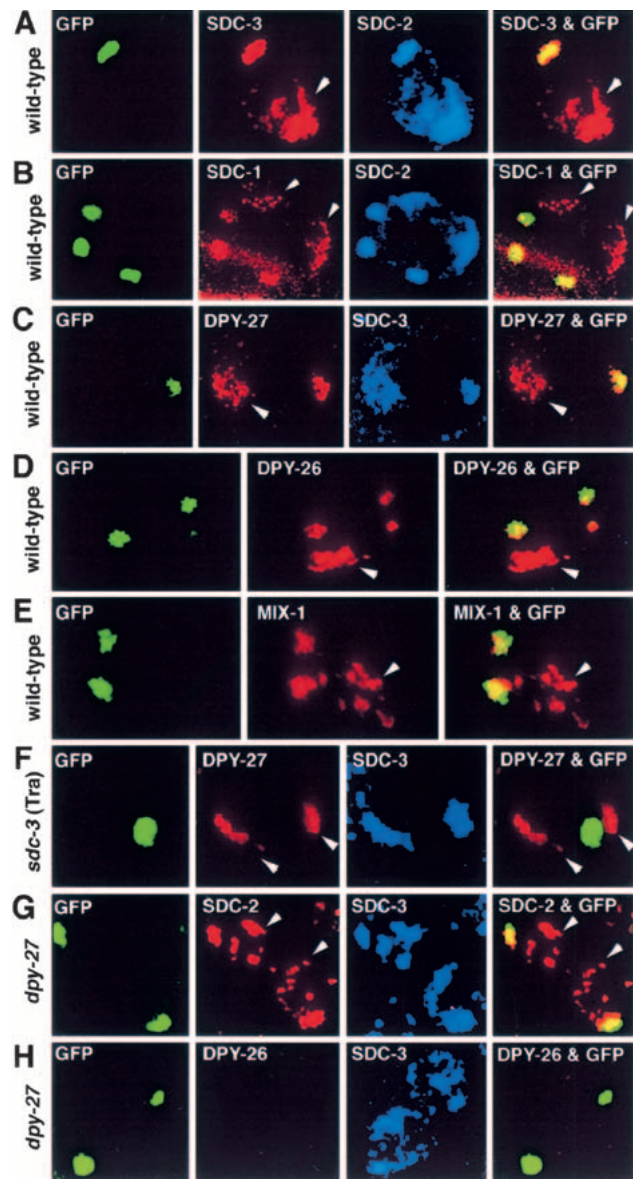


Figure 1. The X-chromosome dosage compensation machinery localizes to *her-1* regulatory regions in vivo. Confocal images of an individual gut nucleus (A–F) or an embryonic nucleus (G,H) from wild-type or mutant [*sdc-3*(Tra) or *dpy-27*] XX animals immunostained with SDC, DPY, or MIX antibodies, as indicated in each panel. The nuclei contain extrachromosomal DNA arrays carrying multiple copies of *her-1* regulatory regions (plasmid pHD25 of Fig. 3A), *lac* operator repeats (*lacO*), and a transgene encoding a LacI–GFP fusion protein. LacI–GFP repressor binding to *lacO* permits array detection by GFP autofluorescence. Colocalization (yellow) between arrays (green) and antibodies (red) in the merged images (right panels) showed association of the protein with *her-1* regulatory sequences. Arrowheads mark the X chromosomes. Consistent with *sdc-3*(Tra) causing derepression of *her-1*, it blocks SDC and DPY proteins from associating with *her-1*.

1999), a sexual transformation that required wild-type *sdc-3* activity. Given the incomplete feminization with SDC-2 alone, we simultaneously overexpressed SDC-2

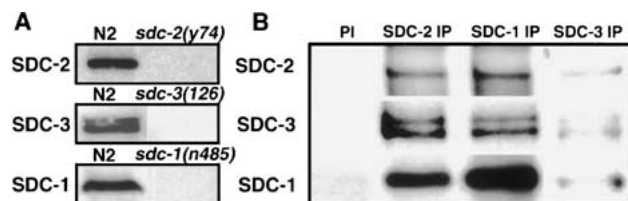


Figure 2. The SDC proteins form a complex in vivo. (A) Detection of SDC proteins in embryonic extracts. Western blots of extracts from wild-type (N2) or *sdc* (null) mutant embryos carrying a deletion or nonsense mutation in the *sdc* gene were probed with the SDC antibody indicated on the left. Proteins of 250 kD, 240 kD (a doublet), and 140 kD were detected by SDC-2, SDC-3, or SDC-1 antibodies, respectively, in wild-type but not *sdc* (null) extracts, showing antibody specificity. (B) Antibody to any one SDC protein coimmunoprecipitated all three SDC proteins. Coimmunoprecipitations were performed with each SDC antibody on wild-type embryonic extracts. The coimmunoprecipitated material was separated by SDS-PAGE and immunoblotted with the antibody indicated on the left. (PI) Preimmune sera for SDC-1 antibody, (IP) immunoprecipitation.

with either SDC-1 or SDC-3 to assess their combined contributions toward hermaphrodite development. Overexpression of only SDC-1 (data not shown) or SDC-3 (Davis and Meyer 1997) failed to feminize XO animals. However, overexpression of both SDC-2 and SDC-1 greatly enhanced the XO feminization, causing ~88% of these XO animals to be sexually transformed (Table 1). Nearly all were self-fertile, in contrast to transformed XO animals that expressed only SDC-2. Increasing the level of SDC-3 in XO animals, as verified by antibody staining, did not enhance the feminization caused by SDC-2 (31% feminization with both proteins), indicating that SDC-3 was not limiting (Table 1). The synergy between SDC-1 and SDC-2 in feminizing XO animals in an SDC-3-dependent manner provides functional evidence that all three SDC proteins act together to repress *her-1* directly.

SDC proteins recruit the X-chromosome dosage compensation complex to *her-1*

Because SDC-2 and SDC-3 play pivotal roles in assembling the dosage compensation complex onto X chromosomes (Chuang et al. 1996; Davis and Meyer 1997; Dawes et al. 1999), we asked whether SDC proteins recruit this complex to *her-1*. The dosage compensation complex includes the dosage compensation-specific protein DPY-27 and the dual-function proteins DPY-26 and MIX-1, which also act in meiosis and mitosis, respectively (Chuang et al. 1996; Lieb et al. 1996, 1998). The dosage compensation proteins resemble components of the widely conserved condensin complex, which drives mitotic chromosome condensation in vitro, implying that regulation of X-chromosome expression involves modulation of chromatin structure (Koshland and Strunnikov 1996; Hirano 2000). All dosage compensation proteins except SDC-2 require SDC-3 for their localization to the X chromosome (Chuang et al. 1996; Davis and Meyer 1997), and SDC-3, in turn, requires SDC-2 for its localization to the X chromosome (Davis and Meyer 1997). SDC-2 can localize to the X chromosome without other dosage compensation proteins (Dawes et al. 1999), suggesting that it recognizes the X chromosome and confers chromosome specificity to dosage compensation. Not all of the dosage compensation components appear essential for *her-1* repression, because the rare *dpy-26*, *dpy-27*, or *dpy-28* XX mutants that escape lethality develop as hermaphrodites (Plenefisch et al. 1989). Therefore, discovery of the complete dosage compensation machinery on *her-1* would show that SDC-2 targets this machinery to the chromatin it binds.

DPY-26, DPY-27, and MIX-1 all colocalized with SDC proteins on both *her-1* arrays and X chromosomes (Fig. 1C–E). Furthermore, localization of the three dosage compensation proteins to *her-1* was dependent on SDC proteins because the *sdc-3*(Tra) mutation disrupted the localization to *her-1* but not to the X chromosome (Fig. 1F; data not shown). The localization of SDC-2 and SDC-3 was not dependent on DPY-27 (Fig. 1G), but DPY-

Table 1. *sdc-1* enhances *sdc-2* in feminizing XO animals

Maternal genotype ^a	Paternal genotype ^a	Extent of XO feminization ^b	No. of XO males	No. of XO intersexes ^c	No. of XO hermaphrodites
<i>dpy-27</i> ^d	<i>dpy-27; yIs30[dpy-30::sdc-2(+)]</i> ^d	36% ^d	408 ^d	31 ^d	200 ^d
<i>dpy-27; yIs3[sdc-3(+)]</i>	<i>dpy-27; yIs30[dpy-30::sdc-2(+)]</i>	31%	84	1	36
<i>dpy-27; yEx31[sdc-1(+)]</i>	<i>dpy-27; yIs30[dpy-30::sdc-2(+)]</i>	88%	23	5	162

^aMaternally rescued *dpy-27*(y167) *unc-32*(e189) III; *lon-2*(e678) X hermaphrodites without transgenic arrays or with transgenic arrays overexpressing either *sdc-3* (from the integrated array *yIs3[sdc-3(+)]*; *rol-6*(d)) or *sdc-1* (from the unintegrated array *yEx31[sdc-1(+)]*; *rol-6*(d)) were crossed to *dpy-27*(y167) III; *him-8*(e1489) IV; *unc-76*(e911) V males overexpressing *sdc-2* (from the integrated array *yIs30[dpy30::sdc-2(+)]*; *unc-76*(+)). Overexpression of SDC-1, SDC-2, and SDC-3 was verified in these lines through antibody staining. Passage of SDC-2 arrays through escaper males did not diminish SDC-2 expression. Lon non-Unc (XO) cross progeny were scored for sexual phenotype. XO embryos that are forced to express SDC proteins activate dosage compensation and die from low levels of X chromosome products. We therefore used the *dpy-27* dosage compensation mutation to restore viability and permit assessment of sexual phenotype.

^bCalculated as [(no. of XO hermaphrodite progeny + no. of XO intersex progeny)/(total no. of XO progeny)] × 100.

^cAnimals with both male and hermaphrodite characteristics.

^dFrom Dawes et al. (1999).

26 required DPY-27 for its localization to *her-1* (Fig. 1H), as it does for its localization to the *X* chromosome. Thus, assembly of known dosage compensation components onto *her-1* resembles their assembly onto the *X* chromosome. Moreover, SDC-2 recruits the dosage compensation machinery to its chromatin targets, even though some components may be dispensable for repression.

The SDC/dosage compensation complex associates with three different chromatin targets within her-1

We determined the exact sites within *her-1* that recruit the dosage compensation machinery. Using the extrachromosomal array assay to examine individual 1-kb fragments across *her-1*, we found that SDC-1, SDC-2, SDC-3, DPY-26, DPY-27, and MIX-1 all colocalized with three different regions defined by fragments B, C, and D (Fig. 3A,B). Localization of all proteins was disrupted by an *sdm-3*(Tra) mutation (data not shown), showing that binding was specific.

Fragment B includes the P1 promoter that produces the 1.2-kb functional *her-1* transcript (Trent et al. 1991; Perry et al. 1993). This regulatory region was first implicated in *her-1* repression by a gain-of-function mutation, *her-1*(gf), that partially derepresses *her-1* transcription, causing substantial, but incomplete masculinization of XX mutants (Trent et al. 1988). The location of *her-1*(gf) 2 bp before the transcriptional start site prompted us to test whether *her-1*(gf) interferes with binding of the repression complex (Perry et al. 1994). Indeed, SDC-2, SDC-3, and DPY-27 failed to associate with a fragment (B') harboring the A → T transition of *her-1*(gf), showing that derepression of *her-1* transcription is caused at least in part by disrupting repressor binding (Fig. 3A,B; data not shown). The *her-1*(gf) mutation appears to eliminate rather than reduce SDC binding, because overexpression of SDC-2 failed to suppress the XX masculinization caused by *her-1*(gf) (see Materials and Methods).

Fragments C and D are within the large second intron of *her-1*. Unlike P1, this specific region had not been implicated in *her-1* repression by gain-of-function mutations. However, the partial masculinization of XX animals by *her-1*(gf) compared with the nearly complete masculinization by *sdm-3*(Tra) suggested that SDC-mediated *her-1* repression requires sequences outside the gf region. Moreover, indirect experiments suggested a possible involvement of the second intron in *her-1* repression (Li et al. 1999).

We explored whether the protein–DNA interactions observed with fragments C and D on extrachromosomal arrays also occurred at the endogenous *her-1* gene by performing chromatin immunoprecipitations (ChIP) from lysates of formaldehyde-treated wild-type embryos. SDC-2 antibodies were used to immunoprecipitate the SDC complex with its associated DNA, and the DNA was analyzed for enrichment of *her-1* fragments using primers to regions A–F in separate PCR reactions. Primers flanking *him-1*, a gene on a different chromosome, and genomic fragments just upstream of region A were used as controls. Only DNA from regions C and D was

specifically enriched by threefold to fourfold relative to the negative control (Fig. 4A). In parallel ChIPs performed with SDC-2 or SDC-3 antibodies, only DNA from wild-type, but not *sdm-3*(Tra) lysates was enriched for fragment C (Fig. 4B). This result confirmed the specificity of the ChIP by showing that it correctly reflects the disruption of SDC binding to *her-1* caused by *sdm-3*(Tra). In controls, equivalent levels of region C DNA were detected in PCRs using DNA extracted from wild-type and mutant lysates (Fig. 4B). Likewise, comparable levels of SDC proteins were detected in both lysates with Western blots (Fig. 4C) and IP experiments (Fig. 4D). Together these experiments show that the SDC complex associates with regions C and D in the endogenous *her-1* gene. The inability to detect region B by this assay suggested that region B has a lower capacity for SDC binding than regions C and D, as shown below and hypothesized previously (Li et al. 1999).

Diverse DNA recognition elements reside within the three distinct her-1 chromatin targets

Having shown that fragments B, C, and D are true SDC targets, we defined the DNA sequence requirements for SDC binding more precisely. Of four region B subfragments, only B2 supported significant SDC-2 colocalization (Fig. 3A). However, localization to B2 was less consistent than localization to B, suggesting that strong SDC-2 binding requires more than one discrete element within region B. In contrast, the robust localization to regions C and D was narrowed to a 303-bp fragment (C5) of C and to a 192-bp fragment (D6) of D (Fig. 3A,B). SDC-1, SDC-3, DPY-26, DPY-27, and MIX-1 all colocalized with SDC-2 on these fragments, showing that all the information required for SDC proteins to interact with chromatin and recruit the dosage compensation complex can be specified by 192 bp of DNA (Fig. 3A,B; data not shown) that has been removed from its native chromosomal context.

Very limited similarity in DNA sequences was found between B (region 1) and either C5 (region 2) or D6 (region 3). In contrast, C5 and D5 (a 287-bp fragment that includes D6) share 50% overall identity and a 15-bp stretch (CAAAACTGAGCCTG) of complete identity on the antisense strand of C5 and the sense strand of D5. An exact copy of this element is not found on *X* or elsewhere in the genome. Randomizing the 15-bp element to ACAGACTGCAGATAC (for C5' and D5') or GACAGACGTCAATAC (for D5') prevented SDC-2, SDC-3, and DPY-27 proteins from localizing to arrays with the mutant fragments (Fig. 3A,B; data not shown). The 15-bp repeated sequence is therefore necessary for targeting SDC and DPY proteins to regions 2 and 3. The 15-mer is not sufficient, however, because SDC-2 failed to associate with arrays carrying multiple copies of random DNA and either the 15-mer or a 28-bp element that includes the 15-mer and neighboring common sequences (data not shown). Other *cis*-acting sequences must be essential. Thus, the three DNA elements used to target the dosage compensation machinery to *her-1* are diverse. Ei-

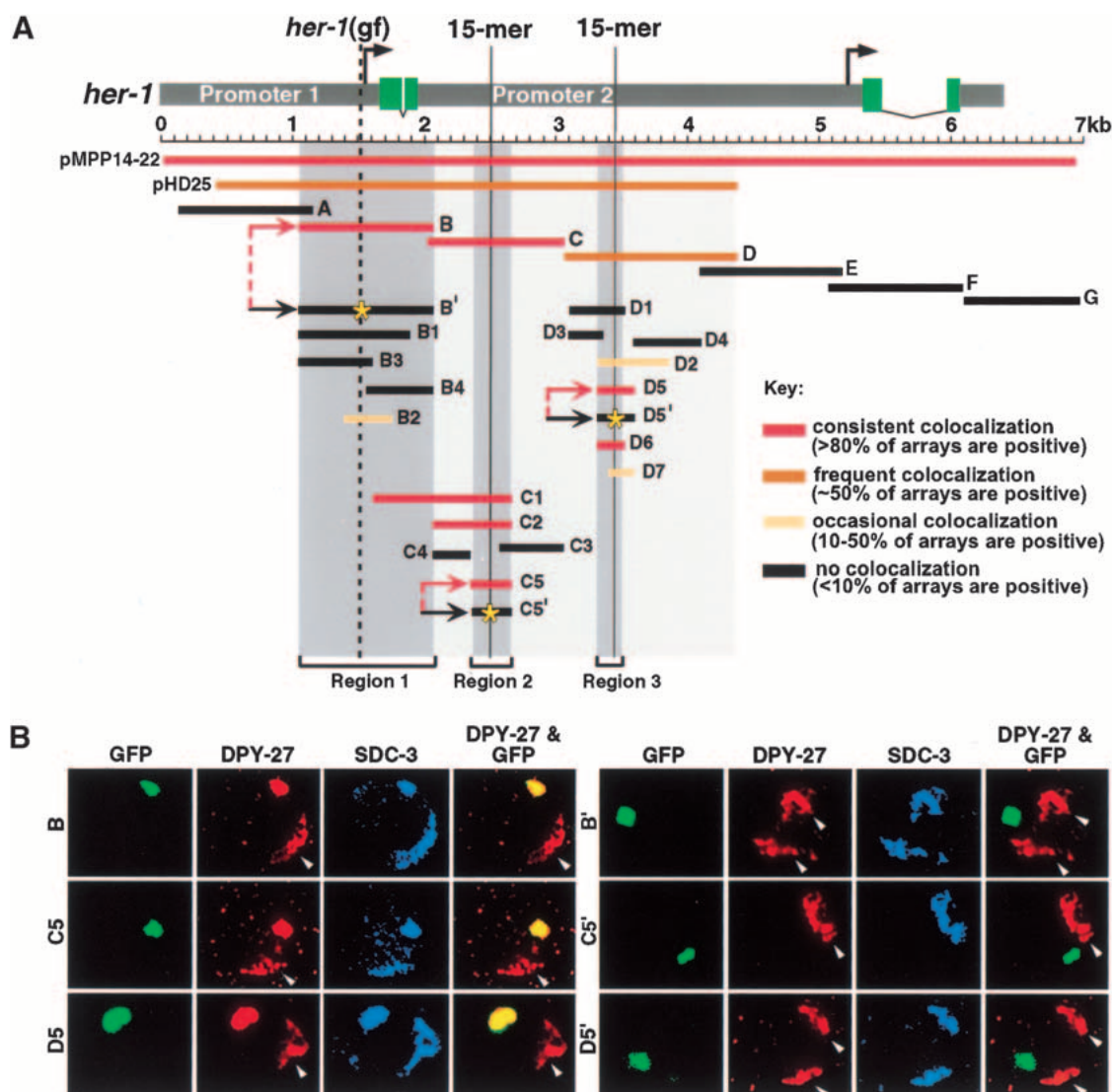


Figure 3. SDC-2 localization to *her-1* is specified by three distinct DNA recognition elements whose binding capacity is disrupted by specific mutations. (A) Schematic of the *her-1* gene and summary of subregions tested for SDC-2 colocalization by the array assay. Transcription from the P1 promoter produces the functional male-specific *her-1* transcript, including four exons (green). A promoter (P2) resides within the second intron of *her-1*. P2 is coregulated with P1 and makes a 0.8-kb transcript of unknown function that includes the last two exons of *her-1* (Trent et al. 1991; Perry et al. 1993). The degree of SDC-2 colocalization with *her-1* regions is shown by color, with the key on the right. The three smallest regions with strong SDC-2 colocalization (region 1 [B], region 2 [C5], and region 3 [D6]) are shown by dark gray shading. C5 and D6 share an identical 15-bp element (solid vertical lines) and 50% overall sequence identity. B has no obvious similarity with C5 or D5 but contains the site of *her-1(gf)* (dashed vertical line). SDC-2 colocalization was completely disrupted by a randomized 15-mer in either C5 or D5 and by the G → A transition of *her-1(gf)* in B (yellow stars in B', C5', and D5'). (B) Confocal images of an individual gut nucleus from a wild-type XX animal bearing GFP-tagged extrachromosomal arrays (green) with either wild-type (B, C5, and D5) or mutant (B', C5', and D5') versions of regions 1-3. Animals were immunostained with antibodies to DPY-27 (red) or SDC-3 (blue). Colocalization between the array and protein appears as yellow in the merged image. Arrowheads indicate X chromosomes.

ther SDC proteins themselves have flexibility in sequence recognition or other cellular components help confer sequence specificity and binding.

Differential recruitment of SDC-2 to *her-1* by individual recognition elements

Identification of mutations that eliminated SDC binding

to individual sites in *her-1* allowed us to assess and correlate in vivo the functional contribution of each site toward overall SDC binding and repression of *her-1*. We introduced the *her-1(gf)* mutation of region 1 and the randomized 15-mers of regions 2 and 3 together or separately into full-length *her-1*-rescuing constructs to test the role of each site. XX animals expressing a single construct from a GFP-tagged extrachromosomal array were

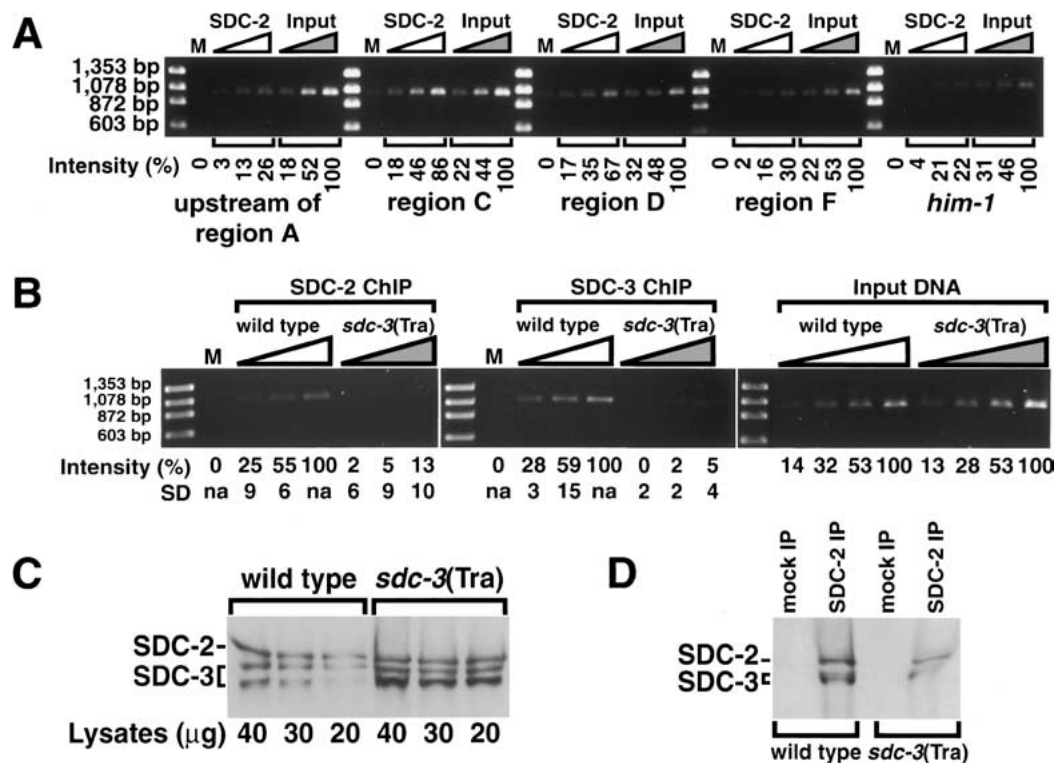


Figure 4. Chromatin immunoprecipitation experiments (ChIP) show the interaction of SDC proteins with the endogenous *her-1* gene. (A) PCR analysis of DNA from a ChIP performed with SDC-2 antibodies and lysates of formaldehyde cross-linked XX embryos. Primer sets specific to *her-1* regions or a control gene (*him-1*) were used for PCRs with mock-precipitated DNA (M) and twofold serial dilutions of SDC-2-precipitated DNA (SDC-2) or input DNA (Input). The intensity of the PCR band produced by each primer pair from IP-enriched DNA was normalized to the corresponding PCR band produced from the highest concentration of input DNA. Regions D and C of *her-1* were specifically enriched above *him-1* control DNA by threefold or fourfold, respectively. (Primers flanking *him-1* produced a PCR product from IP-enriched DNA of 22% normalized intensity, whereas primers flanking regions D and C produced bands of 67% and 86%, respectively, normalized intensity.) (B) PCR analysis with region C primers was performed on twofold serial dilutions of DNA from a ChIP using SDC-2 or SDC-3 antibodies and lysates of formaldehyde-treated wild-type or *sdc-3*(Tra) XX embryos. The intensity of each PCR band was normalized to the intensity of the PCR band made from the highest concentration of IP-enriched DNA from the wild-type lysate. The average intensities and standard deviations were calculated from four sets of PCR analyses on material from two independent ChIP experiments. The specificity of the ChIP protocol was shown by the precipitation of region C DNA from wild-type but not mutant lysates. Similar levels of region C DNA were detected by PCR using twofold serial dilutions of wild-type and *sdc-3*(Tra) input lysates. (C,D) Similar levels of SDC-2 and SDC-3 were detected by Western blot analysis of either (C) whole lysates or (D) SDC-2 IP material from lysates of formaldehyde-treated wild-type and *sdc-3*(Tra) embryos.

examined for the frequency of SDC-2 localization to arrays and for sexual transformation to the male fate, an indicator of transcriptional derepression (see Materials and Methods). The functional significance of a site could then be inferred by comparing the change in SDC localization with the degree of sexual transformation caused by disrupting that site (Fig. 5A).

Regions 2 and 3 had approximately equivalent SDC-2 binding activity in the context of the full-length *her-1* gene, and these regions supported more robust binding than region 1 (Fig. 5A). SDC-2 localized to 90% of arrays carrying a wild-type *her-1* gene. The localization was reduced only slightly, to 85%, by the *her-1*(gf) lesion in region 1, even though this lesion abolished SDC-2 localization to a fragment containing only region 1. The remaining SDC binding must have occurred through regions 2 and 3. Indeed, in transgenes with a wild-type region 1, randomization of either 15-mer decreased

SDC-2 localization to 20%–40%, and randomization of both 15-mers decreased SDC-2 localization to 10%. SDC localization was not significantly reduced by disrupting region 1 on a transgene already mutant for either region 2 or region 3, but was mildly reduced by disrupting region 1 on a transgene mutant for both regions 2 and 3. The comparatively weak SDC binding affinity for region 1 in the context of the full-length *her-1* transgene is consistent with the difficulty in detecting region 1 by ChIP analysis on the endogenous gene.

Complete repression of *her-1* requires the participation of all three SDC-binding regions

Is the strength of SDC binding to a region correlated with effectiveness in repressing *her-1*? Analysis of sexual phenotype in XX animals with wild-type or modified full-length *her-1* transgenes revealed that complete repres-

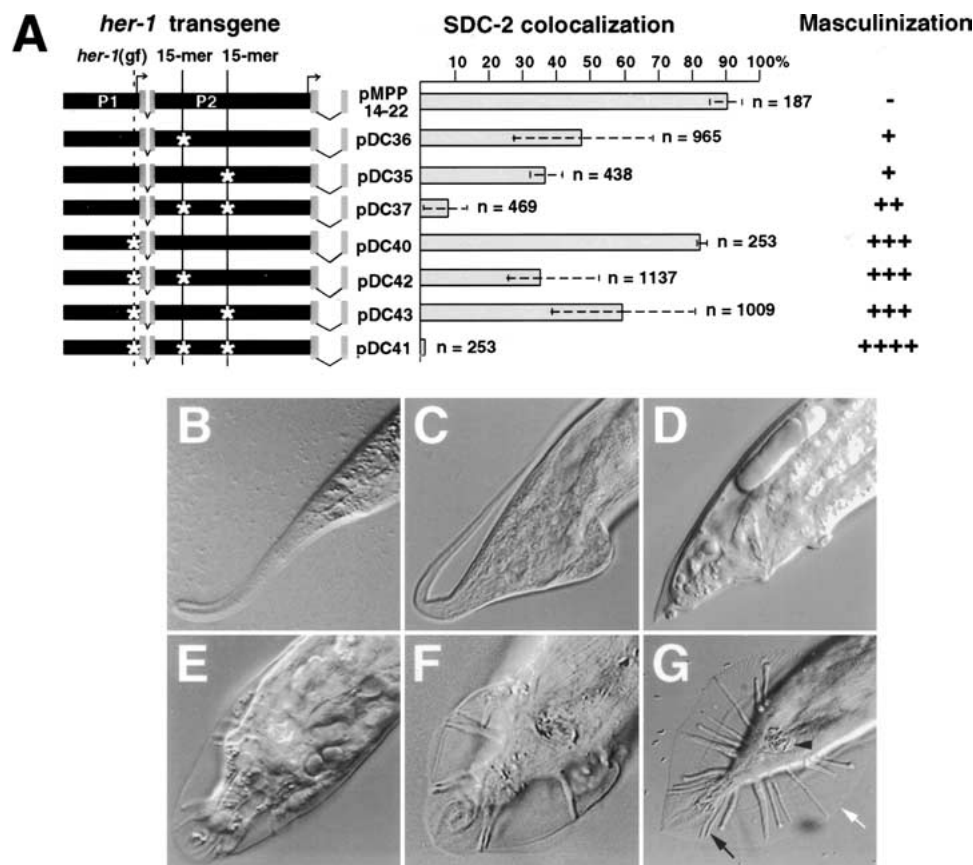


Figure 5. The relative contributions of the three *her-1* recognition elements differ for SDC binding and *her-1* repression. (A) Mutation of specific DNA sequences within full-length *her-1* transgenes disrupts SDC-2 localization to *her-1* and repression of *her-1*. *her-1* constructs included in transgenic arrays are depicted by diagrams on the left, with mutations indicated by white stars. The percent SDC-2 localization to transgenic arrays is represented by gray bars. The standard deviation between lines assayed is represented by a dotted line. (n) Total number of nuclei scored in all lines. Masculinization caused by the full-length *her-1* transgenes was quantified (see Materials and Methods) and then rated as (-) none, (+) weak, (++) moderate, (+++) strong, or (++++). (B-G) Examples of masculinization caused by derepression of *her-1*. DIC photomicrographs of tails from (B) a wild-type XX hermaphrodite, (C-F) XX animals masculinized by full-length *her-1* transgenes, and (G) a wild-type XO male. Lateral views (B-D) and ventral views (E-G). (White arrow) male fan; (black arrow) male sensory rays; (black arrowhead) male spicules.

sion of *her-1* required the participation of all three SDC-binding regions. However, region 1, the weakest in SDC-binding activity, made the greatest single contribution to repression (Fig. 5A-G). Regions 2 and 3 contributed repression activity in the absence of region 1, but repression was less effective than from region 1 (Fig. 5A-G). The degree of *her-1* repression from regions 2 and 3 may be more comparable to the repression of X-linked genes that occurs during dosage compensation.

These conclusions were drawn from the following observations (Fig. 5A-G): XX animals carrying wild-type *her-1* transgenes showed very low levels (-) of masculinization, indicating strong repression. Mutation of either region 2 or 3 caused weak masculinization (+) that was correlated with intermediate disruption of SDC-2 localization. Mutation of both regions 2 and 3 caused moderate masculinization (++), despite causing strong disruption of SDC-2 localization. Finally, mutation of region 1 caused strong masculinization (+++), despite causing only a slight reduction in SDC localization. This mascu-

linization was not enhanced by disrupting only region 2 or 3 but was enhanced by disrupting both regions 2 and 3, causing severe masculinization (++++).

The weak binding of SDC proteins to region 1 on a full-length *her-1* transgene raised the concern that SDC proteins may not mediate repression from region 1. Therefore, we assessed the impact of disrupting SDC binding specifically to region 1 by introducing an *sdc-3*(Tra) mutation into genotypically *her-1*(-) animals carrying full-length *her-1*(+) transgenes with mutations in regions 2 and 3. A twofold to threefold increase in the number of masculinized animals was found, showing that SDC proteins contribute to repression from region 1 in vivo.

The involvement of regions 2 and 3 in SDC-mediated *her-1* repression in vivo is further reinforced by interpreting previous genetic data in the context of the SDC-binding data. Our results show that the *her-1*(gf) mutation eliminates SDC binding to region 1 but not to regions 2 and 3, whereas the *sdc-3*(Tra) mutation severely reduces

SDC binding to all three regions. Thus, the greater degree of masculinization in *XX* animals caused by the *sdc-3*(Tra) mutation (100% of mutants) compared with the *her-1*(gf) mutation (30% of mutants) must result from reduction of SDC binding to regions 2 and 3. Therefore, regions 2 and 3 contribute substantially to SDC-mediated repression of the endogenous *her-1* gene, along with region 1.

Discussion

Gene-specific repression

The work presented here reports a detailed dissection of DNA recognition elements required for SDC binding in vivo and for recruitment of the *C. elegans* dosage compensation complex to the autosomal gene target *her-1*. Within *her-1*, three SDC-binding sites contribute to *her-1* repression: one overlaps the start point of transcription, and two reside within the second intron. Given the locations of the binding sites, more than one mechanism of SDC-mediated repression is likely to control *her-1* transcription. A repression complex bound at any one of the sites could prevent transcription initiation by damping the action of transcriptional activators or by interfering with binding of the basal transcriptional machinery. If such interference were achieved through regions 2 and 3, it would occur over a distance of 1–2 kb and likely be accomplished by a distinct mechanism from that at region 1. Alternatively, repressors bound to regions 2 and 3 might use another strategy to achieve repression, such as impairing transcriptional elongation, altering chromatin structure, or assisting repressor binding to region 1, for example, by looping DNA or acting as repressor storage sites. Through different distances and potentially different mechanisms, the three SDC-binding sites act in concert to repress *her-1* by 20-fold.

Our experiments have raised interesting questions. First, why does region 1 appear to be the most important region for *her-1* regulation in vivo, yet have the lowest affinity for SDC complex binding? A similar observation was made with the regulation of the *Drosophila* master sex-determination gene *Sxl*. The site most effective in activating *Sxl* transcription in vivo had the lowest affinity for the activators (Yang et al. 2001). In the case of *her-1*, overlap between region 1 and the start site of transcription raises the possibility that repression from region 1 is more complex than repression from other regions, possibly involving unidentified repressor molecules, in addition to the SDC proteins. Such additional repressor proteins could act independently of SDC proteins or act in conjunction with them, for example, by assisting SDC binding. Alternatively, the *her-1*(gf) mutation might bolster the inherent rate of transcription initiation, making the task of repression more difficult and the role of region 1 appear greater than the roles of regions 2 and 3.

Second, what is the functional consequence of recruiting the entire dosage compensation complex to *her-1*? Previous work established that the 20-fold repression of *her-1* has different genetic requirements from the two-

fold repression of the *X* chromosome. For example, the *sdc-3*(Tra) mutation prevents assembly of SDC and dosage compensation DPY proteins on *her-1* but not on the *X* chromosome, thereby selectively disrupting *her-1* repression. Moreover, the DPY proteins were thought to play a direct role in repressing only *X*-linked genes but not *her-1*, because rare *dpy XX* mutants that escape lethality develop as hermaphrodites. However, numerous genetic experiments have shown that *dpy-26*, *dpy-27*, or *dpy-28* mutations can affect sexual fate in selective genetic backgrounds (Hodgkin 1987; Miller et al. 1988; Trent et al. 1988; Plenefisch et al. 1989; DeLong et al. 1993). These previous genetic observations, together with our discovery of DPY proteins localized to *her-1* in vivo, suggest that DPY proteins may modulate *her-1* repression directly.

Chromosome-wide repression

The behavior of SDC proteins at *her-1* has important implications for their function in *X*-chromosome-wide gene repression during dosage compensation. SDC proteins have the unexpected ability to associate with diverse DNA elements whose binding capacity can be disrupted by specific mutations. At *her-1*, the sequence and location of each different DNA element specified a distinct level of repression, indicating that repression by the complex is flexible and dependent on the DNA context of its target. The diversity of sequences recognized by the SDC proteins at *her-1* suggests that a specific DNA sequence may not be solely responsible for recruiting the dosage compensation machinery to sites along the *X* chromosome. Although no recognition elements have yet been identified on *X* (Lieb et al. 2000), the regulation of *her-1* suggests that reduction of *X*-linked gene expression has the potential to occur at the level of individual genes, through either short- or long-range mechanisms. Dosage compensation might therefore proceed by combining gene-specific repression strategies with global repression mechanisms acting at the level of chromatin structure, mediated by the condensin-like dosage compensation complex.

Materials and methods

Constructs

Transgenic lines with extrachromosomal arrays carrying *her-1* sequences were created as previously described (Dawes et al. 1999; Lieb et al. 2000) with *lacO* repeat plasmid pSV2-dhFr8.32 (50 µg/mL; Straight et al. 1996), *hsp16-2::lacI-gfp* plasmid pPD49-78 (100 µg/mL), *rol-6(su1006)* marker plasmid pRF4 (100 µg/mL), and *her-1* DNA (50 µg/mL).

The 1-kb regions of *her-1* (designated A–G) and subregions of B, C, and D were PCR-amplified from the full-length *her-1* construct pMPP14-22 (Perry et al. 1993) and blunt-end-ligated into pBluescript. For C4, D1, D2, D3, and D4, PCR products were purified and injected directly into worms. No differences were detected in SDC-2 colocalization assays with extrachromosomal arrays made from *her-1* PCR products or plasmids. Altered full-length *her-1* constructs were made by sequential site-directed mutagenesis of pMPP14-22 using PCR. DNA sequence analysis confirmed the sequence changes.

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Antibodies

SDC-3 antibodies were produced against a GST-SDC-3 fusion protein containing amino acids 1067–1340 of SDC-3 and purified against a thioredoxin-SDC-3 fusion protein containing the same SDC-3 region. SDC-1 antibodies were produced against a his-tagged-SDC-1 fusion protein containing amino acids 615–1034 of SDC-1 and purified against a GST-SDC-1 fusion protein containing amino acids 624–981. SDC-2 antibodies used for immunostaining were raised and affinity-purified against a C-terminal 35-amino-acid peptide plus a CGG linker, CCGDAEESI EDPLDIVEMTLKRALPRSMSPSSKRRMR. Affinity-purified rabbit SDC-2 antibodies used for IP and ChIP reactions were made to a fusion protein containing the first 455 amino acids of SDC-2 and six tandem histidine residues (Dawes et al. 1999). Rat SDC-2 antibodies made to the same fusion protein were used for detecting SDC-2 in the ChIP Westerns. Immunostaining of gravid adults followed (Lieb et al. 2000), except SDC-1 antibodies were incubated with a 1-mg/mL protein lysate from *sdc-1(n485)* embryos, and SDC-2 antibodies were prepared as in Dawes et al. (1999). The *sdc-1(n485)* mutation causes a C → T transition at nucleotide 2634, resulting in a stop codon at amino acid 136. *her-1* extrachromosomal arrays carrying *lacO* and *hsp16-2::lacI-gfp* were identified as in Carmi et al. (1998) and Lieb et al. (2000), except worms were heat-shocked at 33°C for 30 min and allowed to recover at room temperature for 45 min.

Coimmunoprecipitation reactions

Lysates were prepared by sonicating wild-type or mutant embryos in homogenization buffer (50 mM HEPES at pH 7.6, 200 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 5% glycerol, and protease inhibitor cocktail [Calbiochem]). Cellular debris was cleared by centrifugation at 5000g at 4°C for 5 min. Lysates were sonicated further and centrifuged at 20,000g at 4°C for 10 min. The supernatant was incubated with primary antibody at 4°C for 4 h, cleared by centrifugation at 20,000g, and incubated with Protein A Sepharose at 4°C for 1 h. Immunocomplexes bound to Protein A Sepharose were washed with homogenization buffer and eluted with 0.15 M glycine (pH 2.5). Proteins were precipitated with trichloroacetic acid, washed with acetone, and resuspended in Laemmli sample buffer (Laemmli 1970). SDS-PAGE (Novex) and immunoblotting (Bio-Rad) using chemiluminescent detection (ECL, Pharmacia) were performed on immunoprecipitated material.

Quantification of SDC-2 localization to *her-1* arrays

To quantify the localization of SDC-2 to *her-1* arrays (Fig. 3A), an average of 87 array-bearing nuclei (range 25–295) were scored for each construct in every experiment. Multiple independent lines showed consistent results for each construct. For the smallest fragments and their mutant forms, the following number of arrays were scored: B, $n = 78$; B', $n = 40$; C5, $n = 569$; C5', $n = 159$; D5, $n = 169$; and D5', $n = 186$. Colocalization between SDC-1, SDC-3, DPY-26, DPY-27, or MIX-1 on *her-1* arrays was analyzed similarly, with at least 40 worms and >200 gut nuclei examined for each construct.

Effect of SDC-2 overexpression on *her-1(n695gf)* mutants

To assess whether overexpression of SDC-2 suppresses *her-1(gf)* mutants, *yIs30[dpy30::sdc-2(+); unc-76(+)]*; *him-8 IV*; *unc-76 V* survivor males were crossed to *unc-42(e270) her-1(n695gf) V* hermaphrodites, and non-Unc F₁ hermaphrodites were allowed to self-fertilize. Overexpression of SDC-2 from *yIs30* was confirmed by antibody staining. One-hundred Unc-42 F₂ XX adults were scored for sexual phenotype, including masculinization of

tail and soma, sterility, and egg-laying defects, and then picked for single-worm PCR. The presence of *yIs30* was detected using the oligonucleotides HD-43 (CTC GCT GTC AGT GTT TTG TCC TG) and HD-44 (CAT CCA TCT CGA AAT CTC CGA G), which span the *dpy-30-sdc-2* junction in the transgene, to create a PCR product of ~500 bp. Of 100 F₂ animals, 64 carried at least one copy of *yIs30*. Overexpression of SDC-2 from *yIs30* did not rescue any of the sexual phenotypes associated with *her-1(gf)*.

Chromatin immunoprecipitation analysis

We devised a protocol similar to that of Hecht and Grunstein (1999) using worm embryos that were fixed in M9 buffer with 2% formaldehyde at room temperature for 30 min. Excess formaldehyde was quenched and removed with a 0.1 M Tris-HCl (pH 7.5) wash and two M9 washes. Lysates were prepared as described above except ChIP buffer was used (50 mM HEPES-KOH at pH 7.6, 1 mM EDTA, 140 mM KCl, 0.5% NP-40, 10% glycerol, 5 mM DTT). Lysates were precleared against Protein A Sepharose or IgG-sorb (The Enzyme Center). For each ChIP reaction, 3 mg of total protein was incubated with 5 µg of affinity-purified antibodies for 2 h. After clearing nonspecific aggregates by centrifugation at 16,000g, the immunocomplexes were captured with Protein A Sepharose and subjected to four 1-mL ChIP buffer washes at 100 mM KCl and two at 1 M KCl, and two TE (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) washes. The precipitates were eluted with 10 mM Tris-HCl (pH 8) and 1% (w/v) SDS. For protein detection, the immunocomplexes were washed six times with 1 mL of ChIP buffer and eluted with 0.1 M glycine (pH 3). For ChIP analysis, formaldehyde cross links were reversed by incubation at 65°C overnight in 0.2 M NaCl. Proteins were removed by proteinase K digestion and phenol-chloroform extraction. Following ethanol precipitation, the DNA was resuspended in 100 µL of TE. For input DNA control, DNA was extracted from 3 mg of lysates as described above. PCR amplifications (25 cycles with 55°C annealing for 30 sec and 72°C extension for 90 sec in 50 µL of reaction volume) were carried out on 0.75, 1.5, and 3 µL of undiluted SDC precipitated DNA and input DNA diluted 3300-fold. The working concentration for each primer pair was optimized to produce similar amounts of PCR products from the input DNA. For all primers, PCR amplification was always linear in the dilution range of the DNA used. PCR products resolved on an agarose gel were visualized by ethidium bromide staining and quantitated on the Gel-Doc System (Bio-Rad) using the pixel value for each band to calculate relative intensity.

Quantification of *her-1* repression from extrachromosomal arrays

Masculinization of XX animals carrying extrachromosomal arrays of full-length wild-type or mutant *her-1* transgenes was used to evaluate derepression of *her-1*. Egg-laying defects, sterility, male gonad structure, and evidence of male tail formation (Fig. 5C–F) were indicators of masculinization (Trent et al. 1988). Percent masculinization (% Tra) is [(the number of masculinized transgenic animals per line)/(total number of transgenic animals per line)] × 100. Variability in masculinization (% Tra) between lines with the same construct was observed, and all lines generated animals with a range of masculinized tails. Variability may be caused by differential array silencing or stability and a different number of transgenes per array. Therefore, a qualitative rating for masculinization was devised using two criteria: (1) the maximum masculinization (% Tra) value per construct and (2) the degree of masculinization for most animals per construct. The qualitative ratings (shown in parentheses) correlated with maximum masculinization (% Tra): (–) none,

8% Tra; (+) weak, 41%–55% Tra; (++) moderate, 78% Tra, with most animals having tails C and D; (+++) strong, 83%–100% Tra, with most animals having tails C–E; and (+++++) severe, 100% Tra, with most animals having tails E and F. The number of transgenic animals (*n*) scored from all lines of each construct was: pMPP14-22, *n* = 456, 5 lines; pDC35, *n* = 562, 5 lines; pDC36, *n* = 767, 3 lines; pDC37, *n* = 1008, 4 lines; pDC40, *n* = 121, 1 line; pDC41, *n* = 86, 2 lines; pDC42, *n* = 1281, 8 lines; pDC43, *n* = 282, 2 lines.

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A molecular link between gene-specific and chromosome-wide transcriptional repression

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