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Materials and Methods

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Disassembly of Transcriptional Regulatory Complexes by Molecular Chaperones

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Many biological processes are initiated by cooperative assembly of large multicomponent complexes; however, mechanisms for modulating or terminating the actions of these complexes are not well understood. For example, hormone-bound intracellular receptors (IRs) nucleate formation of transcriptional regulatory complexes whose actions cease promptly upon hormone withdrawal. Here, we show that the p23 molecular chaperone localizes in vivo to genomic response elements in a hormone-dependent manner, disrupting receptor-mediated transcriptional activation in vivo and in vitro; Hsp90 weakly displayed similar activities. Indeed, p23 and Hsp90 also disrupted the activities of some non–IR-containing transcriptional regulatory complexes. We suggest that molecular chaperones promote disassembly of transcriptional regulatory complexes, thus enabling regulatory machineries to detect and respond to signaling changes.

Many physiological signal transduction pathways culminate in selective modulation of specific gene transcription rates. For example, glucocorticoid or thyroid hormones in the bloodstream enter target cells and associate with the glucocorticoid receptor (GR) or thyroid hormone receptor (TR), respectively. These receptor-hormone complexes bind selectively to specific genomic sites termed hormone response elements (HREs) and trigger formation of large, heterotypic transcriptional regulatory complexes, which in turn activate or repress transcription from nearby promoters (*1*, *2*). The effects of hormonal signaling are quite rapid: Rates of transcription at inducible genes increase with a half-life ($t_{1/2}$) of ~5 to 10 min (*3*, *4*). Similarly, hormone withdrawal leads promptly to hormone release from the receptor and cessation of activation (*5*).

How does an intracellular receptor (IR), bound to hormone and in the cell nucleus as part of a large regulatory complex, detect and respond to a decline in extracellular hormone levels? Recent studies suggest that certain factors, when assembled into regulatory complexes, may become targets for proteasomal degradation (*6*). However, IRs do not appear to be degraded upon hormone withdrawal; indeed, unliganded IRs commonly display longer intracellular half-lives than their liganded counterparts (*7*, *8*). Rather, we have proposed that IR-containing regulatory complexes may be actively and reversibly disassembled by molecular chaperones (*9*).

We tested directly whether the p23 molecular chaperone may be involved in the disassembly of transcriptional regulatory complexes, by (i) assessing the actions of p23 on functional regulatory complexes in vitro, (ii) developing a general approach to determine in vivo the effects of chaperone components on regulatory complexes containing IRs or non-IR regulators such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP1), and (iii) monitoring the recruitment of chaperone components to natural response elements in vivo.

The effect of purified p23 on TR-containing regulatory complexes and TR-mediated transcriptional activation in vitro is shown in Fig. 1 (*10*). In these experiments, receptor-DNA complexes were formed on two thyroid response element (TRE)–containing templates: the four-spaced direct repeat (DR_4) TRE favors TR/RXR heterodimer binding, whereas the palindromic TRE (TREpal) favors TR homodimer binding. In one experiment, we added p23 to the receptor-DNA complexes before the addition of HeLa nuclear extract (Fig. 1A). On the basis of our findings with purified components, we predicted that p23 should dissociate TR/RXR or TR from the template, unless factors in the extract reverse or preclude that process, and that transcriptional activation by the receptor should be reduced. Indeed, receptor-mediated activation, but not basal transcription (as shown by a control template, ADHp, lacking response elements), declined as a function of increasing p23.

In a second experiment, we addressed whether p23 could affect fully elaborated transcriptional regulatory complexes, which may correspond to its substrates in vivo. Specifically, we examined the effect of adding p23 to reactions in which receptor-DNA complexes were first preincubated for 30 min with nuclear extract (Fig. 1B). Under these conditions, fully functional regulatory complexes should assemble at the response elements, and as a result, competent preinitiation complexes should form at the promoter. We used Sarkosyl, which inactivates RNA polymerase II that is not in competent preinitiation complexes (*11*), to confirm that such complexes were indeed formed during the preincubation (Fig. 1B; compare Sa with Sb). We found that added p23 abrogated transcriptional activation by these preformed complexes, suggesting that p23 is a limiting factor for disassembly of functional regulatory complexes in vitro.

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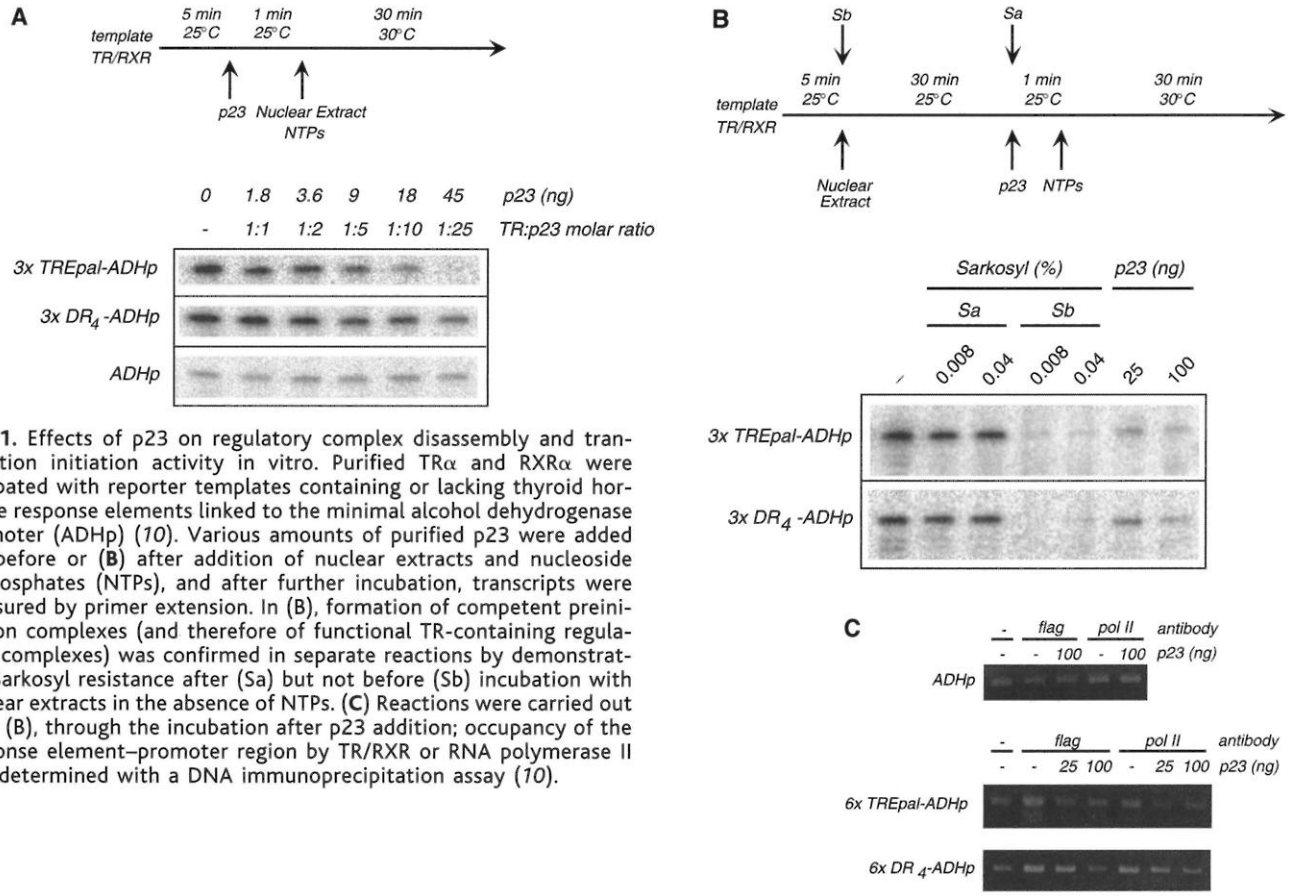

Fig. 1. Effects of p23 on regulatory complex disassembly and transcription initiation activity in vitro. Purified TRα and RXRα were incubated with reporter templates containing or lacking thyroid hormone response elements linked to the minimal alcohol dehydrogenase promoter (ADHp) (*10*). Various amounts of purified p23 were added (**A**) before or (**B**) after addition of nuclear extracts and nucleoside triphosphates (NTPs), and after further incubation, transcripts were measured by primer extension. In (B), formation of competent preinitiation complexes (and therefore of functional TR-containing regulatory complexes) was confirmed in separate reactions by demonstrating Sarkosyl resistance after (Sa) but not before (Sb) incubation with nuclear extracts in the absence of NTPs. (**C**) Reactions were carried out as in (B), through the incubation after p23 addition; occupancy of the response element–promoter region by TR/RXR or RNA polymerase II was determined with a DNA immunoprecipitation assay (*10*).

Finally, we developed a DNA immunoprecipitation assay to assess promoter occupancy by transcription factors in vitro (*10*). Using this assay, we tested whether p23 could disassemble the TR/RXR regulatory complexes and perhaps destabilize RNA polymerase II preinitiation complexes at a TRE-linked promoter (Fig. 1C). We found that p23 reduced the level of TR/RXR binding to TREs, implying that p23, perhaps together with other chaperone components in the extracts, disassembled TR/RXR-containing regulatory complexes from the reporter template in vitro. Moreover, maybe as a secondary consequence of regulatory complex disassembly, RNA polymerase II preinitiation complexes also were disassembled in the same reactions.

We next investigated whether the activities we observed for p23 in vitro might also be detected in vivo. Such studies are complicated by the high endogenous expression levels and by the numerous potential chaperone substrates that might influence transcriptional activation. To address these problems, we devised an approach to selectively elevate, close to response elements of choice, the local concentration of candidate molecular chaperones. We constructed "linked response element" (RE) reporters containing two hormone response elements (HRE, either glucocorticoid or thyroid hormone) adjacent to two GAL4 binding sites (Fig.

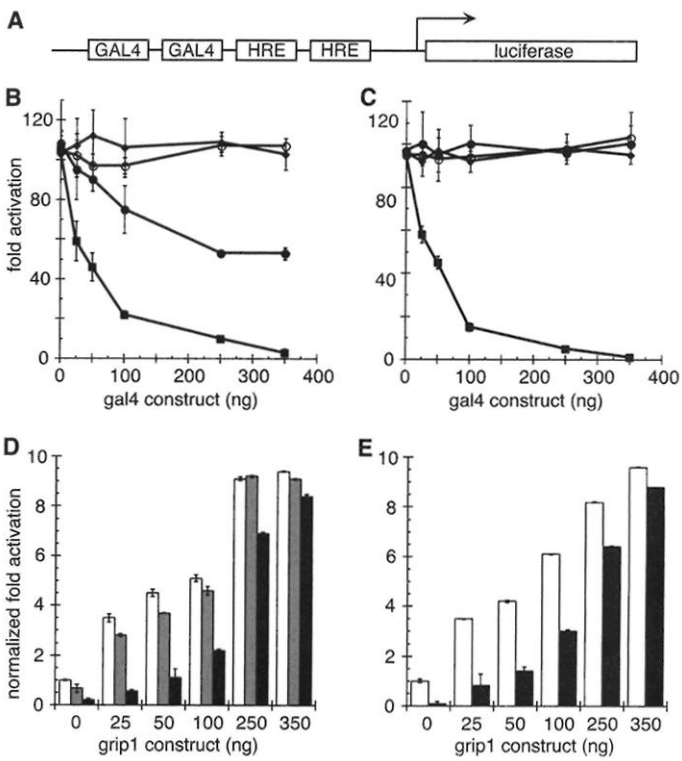

Fig. 2. (**A** to **C**) Effects of targeted localization of molecular chaperones near hormone response elements (HREs) in vivo. HTC rat hepatoma cells were transiently transfected (*10*) with "linked response element" (RE) reporters (A) that contain REs for GR (B) or TR (C) and drive expression of luciferase [$(TRE)_2(GAL4)_2$ or $(GRE)_2(GAL4)_2$, respectively]; in conjunction with the $(TRE)_2(GAL4)_2$ reporter, an expression plasmid for TR (pSG5-hTRβ; 10 ng) was cotransfected. In addition to these plasmids, expression constructs for the Gal4 DNA binding domain (○), Gal4-p23 (■), Gal4-Hsp90 (●), or Gal4-Hsp70 (◆) were cotransfected. (**D** and **E**) The effect of the coactivator GRIP1 on GR (D) and TR (E) activity. HTC cells were transfected with the indicated amounts of an expression plasmid for GRIP1 (pSG5-GRIP1), the appropriate reporter constructs. Transcriptional activity was assessed in the absence (white bars) or presence of cotransfected expression constructs (100 ng) for Gal4-Hsp90 (gray bars) and/or Gal4-p23 (black bars).

2A); in addition, we prepared expression vectors for fusion proteins between the Gal4 DNA binding domain and human p23, Hsp90, or Hsp70. Hsp90 and Hsp70, together with p23, are established aporeceptor components that appear to associate in heteromeric complexes (*12*).

Expression of Gal4-p23 reduced GR (Fig. 2B; ~35-fold) and TR (Fig. 2C; ~100-fold) transcriptional activation (*10*). In contrast, Gal4-Hsp90 selectively but modestly (~twofold) reduced GR activation, and Gal4-Hsp70 expression had no apparent effect on either TR or GR. Thus, forced localization of p23 to a HRE virtually abrogates receptor-induced activation, consistent with p23-driven regulatory complex disassembly.

Notably, the Gal4-chaperones did not alter basal promoter activities, whereas expression of a Gal4–repression domain chimera (Gal4-Mad) produced a threefold reduction in basal activity (*13*). Thus, the chaperones are not general corepressors; instead, they selectively reduce the efficacy of the regulatory complexes in our assays, as would be expected if they selectively promote disassembly of those complexes.

Freeman *et al.* (*14*) demonstrated that a peptide corresponding to the TR interaction surface (i.e., NR box 2) of the GRIP1 coactivator could associate with TR-TRE complexes in vitro and stabilize them against p23-mediated dissociation. Alternate stepwise addition of p23 and the GRIP1 peptide into these reactions reveals a competitive equilibrium in vitro, implying that p23, in addition to its capacity to dissociate receptors from DNA, also dissociates the coactivator fragment from receptors. If this in vitro activity reflects the actions of these components in vivo, then overexpression of full-length GRIP1 might relieve the effects of localized chaperones on receptor activity. Indeed, we found that high levels of cotransfected GRIP1 expression vector (*15*) mitigated Gal4-p23 inhibition of TR and GR activity in vivo (Fig. 2, D and E). Similarly, the twofold reduction in GR activity conferred by Gal4-Hsp90 was relieved by GRIP1 overexpression. GRIP1 overexpression also abrogated the ~fourfold inhibition of receptor activity conferred by overexpressed intact p23 (*13*, *14*). We suggest that p23, and to a lesser extent Hsp90, can disrupt receptor interactions in vivo, both with response elements and with coactivators.

If chaperones recognize natural regulatory complexes and mediate their disassembly, components of the molecular chaperone complex might be detectable at functional response elements in vivo. We therefore examined the glucocorticoid response elements (GREs) associated with two liver-specific glucocorticoid-inducible genes in rat hepatoma HTC cells, tyrosine aminotransferase (TAT) and tryptophan oxygenase (TO); regulatory complexes assemble and function at these GREs only in the presence of hormone. Using the chromatin immunoprecipitation assay (*10*), we observed increased occupancy by GR at both GREs after addition of dexamethasone (Fig. 3); the extent of these increases was about five- to sevenfold greater than the average change measured at two control segments that lack functional GREs, namely, a 5′-flanking region upstream of the TAT GRE, and the HSP60 promoter. Both p23 and Hsp90 also localized to the response elements in a hormone-dependent manner, whereas Hsp70 was not detected (Fig. 3). Thus, p23 and Hsp90 are selectively recruited to functional GR-containing regulatory complexes in vivo.

To determine whether chaperones also influence the activities of regulatory complexes that do not include members of the intracellular receptor family, we constructed variants of our linked RE reporter plasmids (Fig. 2A) in which the HREs were replaced by two tandem κB or AP1 response elements. The individual reporters were then introduced by transient transfection into A549 human lung epithelial cells, where cognate factors NF-κB and AP1 can be activated by tumor necrosis factor–α (TNF-α) and phorbol 12-myristate 13-acetate (PMA), respectively (*10*). Cotransfection of Gal4-p23 or Gal4-Hsp90 reduced by ~sevenfold or ~threefold, respectively, the TNF-α–induced NF-κB activity; Gal4DBD and Gal4-Hsp70 had no apparent effect (Fig. 4A). In contrast, AP1 activity was affected only by Gal4-p23 (~fivefold reduction; Fig. 4B). Although further studies will be needed to test the functional relevance of these observations, they suggest that chaperones may act broadly to disassemble transcriptional regulatory complexes.

Given the physical properties of regulatory complexes, it seems likely that their disassembly requires expenditure of energy. Some minimal reactions, such as dissociation of purified receptors from DNA, or of a coactivator peptide from a receptor, can be

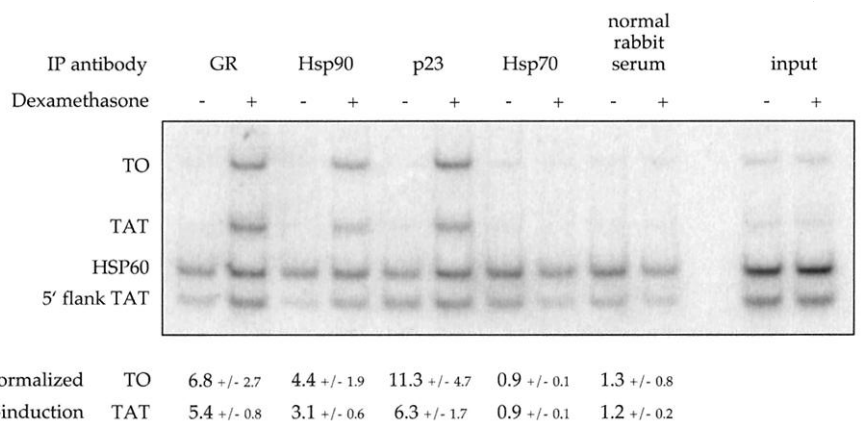

Fig. 3. Occupancy of GREs by GR and molecular chaperones in vivo. Rat hepatoma HTC cells were untreated, or were incubated for 20 min at 37°C with 100 nM dexamethasone. Binding of various factors to two functional GREs that mediate dexamethasone induction of tryptophan oxygenase (TO) and tyrosine aminotransferase (TAT) in liver cells (S2, S3) was determined with the chromatin immunoprecipitation assay (*10*). As controls, apparent occupancies at two non–GRE-containing fragments were monitored: a region ~7.3 kb upstream of the TAT gene (5′ flank TAT; ~2.5 kb upstream of the nearest functional GRE) and the promoter region of the HSP60 gene. The presented values were normalized to the controls and are averages (mean ± SD) from three independent assays.

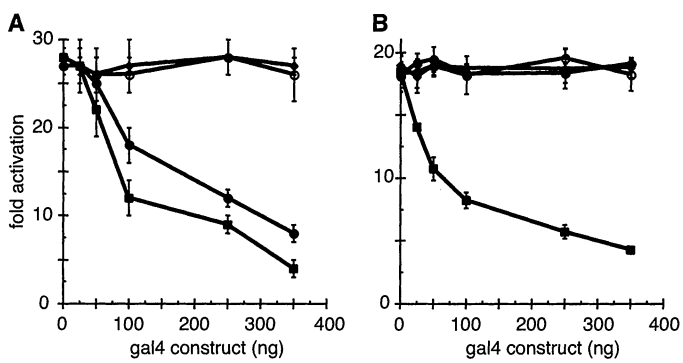

Fig. 4. Effects of molecular chaperones on activities of non-IR regulators. A549 cells were transiently transfected (*10*) with κB (**A**) or AP1 (**B**) linked RE reporters [$(\kappa B)_2(GAL4)_2$ or $(AP1RE)_2(GAL4)_2$, respectively] driving the expression of luciferase; expression constructs for Gal4 DNA binding domain (○), Gal4-p23 (■), Gal4-Hsp90 (●), or Gal4-Hsp70 (◆) were cotransfected. Endogenous NF-κB or AP1 was activated by 6-hour incubations with TNF-α (2.5 ng/ml) or PMA (10 ng/ml), respectively.

catalyzed by the non-ATPase (adenosine triphosphatase) chaperone p23 (*12*). Perhaps it is notable in this regard that we have not observed release of hormone in those reactions. We have not yet addressed whether the disassembly of intact complexes requires energy, but at least two components of molecular chaperone complexes, Hsp90 and Hsp70, are ATPases, and it is thought that Hsp90 and p23 can associate directly (*12*).

Purified hormone-IR complexes are stable, consistent with crystallographic studies revealing that the ligands are buried within the hydrophobic core of the ligand-binding domains, contacted by 15 to 20 amino acid side chains (*16*). In contrast, hormones are released from receptors in vivo with half-lives of a few minutes (*17*). Similarly, regulatory complexes commonly assemble cooperatively and stably in vitro (*1*, *18*), whereas they are exceptionally dynamic in vivo (*19*, *20*), turning over every few seconds (*21*). We propose that molecular chaperones resolve these apparent discrepancies by fully disassembling regulatory complexes in vivo, which may include release of the hormone when the complexes contain intracellular receptors. Notably, continuous disassembly of regulatory complexes would enable IRs and other regulators to sense and respond efficiently to fluctuations in hormone levels (*9*). This principle could extend similarly to other classes of signals that affect regulatory factor activity, stability, or localization.

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Regulation of the Different Chromatin States of Autosomes and X Chromosomes in the Germ Line of *C. elegans*

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The Maternal-Effect Sterile (MES) proteins are essential for germline viability in *Caenorhabditis elegans*. Here, we report that MES-4, a SET-domain protein, binds to the autosomes but not to the X chromosomes. MES-2, MES-3, and MES-6 are required to exclude MES-4 and markers of active chromatin from the X chromosomes. These findings strengthen the emerging view that in the *C. elegans* germ line, the X chromosomes differ in chromatin state from the autosomes and are generally silenced. We propose that all four MES proteins participate in X-chromosome silencing, and that the role of MES-4 is to exclude repressors from the autosomes, thus enabling efficient repression of the Xs.

The phenomenon of X-chromosome dosage compensation is fairly well understood in somatic cells (*1*). How the germ line modulates gene expression from the X chromosomes is less clear. Recent findings suggest that the X chromosomes in *C. elegans* are globally repressed during most of germline development (*2*, *3*). The four *C. elegans* MES proteins are prime candidates for regulating this repression. The *mes* genes display a maternal-effect sterile phenotype that is highly sensitive to X-chromosome dosage; among the progeny of *mes/mes* mothers, XX animals undergo germline degeneration and lack gametes, whereas XO animals are usually fertile (*4*, *5*). MES-2 and MES-6 are homologs of Enhancer of zeste and Extra sex combs, both members of the Polycomb group of transcriptional repressors (*6*, *7*). MES-3, which has no known homologs, forms a complex with MES-2 and MES-6 (*8*, *9*).

Cloning and sequencing the *mes-4* gene [fig. S1 (*10*)] revealed that it encodes a 3.2-kb transcript that is enriched in the germ line (*11*). The predicted MES-4 protein (898 amino acids in length) is similar in sequence and motif organization to the predicted *Drosophila* protein CG4976 (*12*), the mouse protein NSD1 (*13*), and the human protein MMSET (*14*) [fig. S2 (*10*)]. All three proteins share three plant homeodomain (PHD) fingers, which mediate protein-protein interactions (*15*), and a SET domain with flanking cysteine-rich regions. The SET domain, common to many chromatin-binding proteins, mediates protein-protein interactions (*16*) and in some cases [e.g., SUV39H1 (*17*)] methylates lysine residues of histone H3. MES-4 shows sequence similarity to SUV39H1 within the SET domain [fig. S2 (*10*)].

On the basis of immunofluorescence staining (*10*), MES-4 is localized to nuclei and associated with chromosomes. MES-4 is present in the distal, mitotic region of the germ line, barely detectable in the early- to mid-pachytene region, and up-regulated in later pachytene and in oocytes (Fig. 1A). In embryos, MES-4 is present in both somatic and germline nuclei until the 80- to 100-cell stage (Fig. 1, B to D). Subsequently, MES-4 staining diminishes in somatic cells but persists in the primordial germ cells Z2 and Z3 (Fig. 1, E and F), in accordance with the requirement for MES-4 to protect germline viability.

Staining in one-cell embryos revealed that one chromosome of each parental set of six lacks MES-4 (Fig. 2A). In four-cell embryos two chromosomes in each diploid nucleus lack MES-4 (Fig. 2B). The sensitivity of the Mes phenotype to X-chromosome dosage suggested that the unstained chromosome is the X. The following results verify this prediction: (i) a single unstained chromosome is observed in XO embryos (Fig. 2C); (ii) three unstained chromosomes are observed in XXX embryos (Fig. 2D); and (iii) the X portion of an X:autosome translocation is not stained (Fig. 2E).

MES-4 is also restricted to the autosomes in the adult hermaphrodite germ line. In the distal

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