Dimerization of Doublesex Is Mediated by a Cryptic Ubiquitin-associated Domain Fold

IMPLICATIONS FOR SEX-SPECIFIC GENE REGULATION

Male- and female-specific isoforms of the Doublesex (DSX) transcription factor regulate somatic sexual differentiation in Drosophila. The isoforms (DSX\textsuperscript{M} and DSX\textsuperscript{F}) share an N-terminal DNA binding domain (the DM motif), broadly conserved among metazoan sex-determining pathways. DM-DNA recognition is enhanced by a C-terminal dimerization domain. The crystal structure of this domain, determined at a resolution of 1.6 Å, reveals a novel dimeric arrangement of ubiquitin-associated (UBA) folds. Although this \(\alpha\)-helical motif is well characterized in pathways of DNA repair and subcellular trafficking, to our knowledge this is its first report in a transcription factor. Dimerization is mediated by a non-canonical hydrophobic interface extrinsic to the putative ubiquitin binding surface. Key side chains at this interface, identified by alanine scanning mutagenesis, are conserved among DSX homologs. The mechanism of dimerization is thus unrelated to the low affinity domain swapping observed among ubiquitin-associated CUE domains. The unexpected observation of a ubiquitin-associated fold in DSX extends the repertoire of \(\alpha\)-helical dimerization elements in transcription factors. The possibility that the ubiquitination machinery participates in the regulation of sexual dimorphism is discussed.

Sexual differentiation in Drosophila is regulated by the X:autosome ratio and a sex-specific RNA-splicing pathway (Fig. 1A; Ref. 1). A principal target is doublesex (dsx), expression of male- or female-specific transcription factors (DSX\textsuperscript{M} and DSX\textsuperscript{F}) directs most aspects of somatic sexual differentiation (2). The DSX isoforms are encoded by mRNAs sharing the first three exons; the C-terminal segment of DSX\textsuperscript{M} is encoded by exon 4, whereas that of DSX\textsuperscript{F} is encoded by exons 5 and 6. Male and female isoforms are, thus, identical for the first 397 residues but differ thereafter (Fig. 1B; Ref. 2). Common elements include an N-terminal DNA binding domain, the DM motif (3). Mutations in the DSX DM domain (a non-classical Zn module (4)) block DNA binding in association with intersexual phenotypes (5, 6). Broad conservation of the DM domain in metazoan proteins related to sexual differentiation suggests that mechanisms of sexual dimorphism are in part universal (3).

The C-terminal domains of DSX\textsuperscript{M} and DSX\textsuperscript{F} (CTDF and CTD\textsuperscript{F}) are conserved among insect homologs. The domains contain a common dimerization element and sex-specific extensions proposed to mediate recruitment of transcriptional co-regulatory factors (7, 8). The strength of dimerization (as inferred from DNA binding) is <1 nM (9). Such dimerization enhances specific DNA binding (9); a mutation in CTD\textsuperscript{F} that blocks dimerization is associated with intersexual development (7). The dimer does not contain a recognizable structural motif but has been predicted to form a coiled-coil (10). In this article we describe the crystal structure of a dimeric fragment of CTD\textsuperscript{F} at 1.6 Å resolution and its scanning mutagenesis in a yeast two-hybrid (Y2H) system. The polypeptide (designated CTD\textsuperscript{F}-p) spans residues 350–412 and so contains both shared and sex-specific sequences. Surprisingly, the structure reveals a novel dimeric arrangement of ubiquitin-associated-domain (UBA) folds (Protein Data Bank code 1ZV1). To our knowledge, this \(\alpha\)-helical motif, although widely conserved among pathways regulating DNA repair and subcellular trafficking, has not previously been found in a transcription factor. Dimerization is mediated by an extensive non-polar interface conserved among DSX homologs. The structure of CTD\textsuperscript{F}-p extends the family of transcription-related \(\alpha\)-helical dimerization elements and raises the possibility that the ubiquitination machinery participates in the regulation of sexual dimorphism.

EXPERIMENTAL PROCEDURES

Protein Crystallization—CTD\textsuperscript{F}-p (65 residues; GS followed by DSX\textsuperscript{F} residues 350–412) was designed based on Y2H studies (7, 10) and expressed in Escherichia coli (strain B834(DE3)pLysS) as a thrombin-cleavable fusion protein and purified as described (11). For selenomethionine labeling, the protein was expressed in M9 minimal medium containing 50 mg/liter selenomethionine and all other amino acids (except methionine) at 40 mg/liter (11). Crystals were obtained by hanging-drop vapor-diffusion in 4-\(\mu\)l drops containing equal volumes of protein stock (12 mg/ml) and reservoir buffer (1.8 M ammonium sulfate and 7% 2-propanol).

Data Collection and Structure Determination—Native data were collected at Advanced Photon Source beamline 14BM at 100 K. Single-wavelength anomalous dispersion data were collected at NSLS beamline X9B at the selenium peak (0.9788 Å). Data collection statistics are given in TABLE ONE. Data were integrated and scaled with HKL2000 (12). Substructure determination and phasing were accomplished with the SHELX suite of programs (13). Initial model building employed WARP/ARP (14). Additional rounds of model building were performed.
Structure of Doublesex

using O (15). Initial refinement was accomplished using CNS, applying non-crystallography symmetry, overall B-factor corrections, and bulk solvent corrections (16). Final rounds of refinement were performed with the CCP4 program REFMAC5 (17). Accuracy of the model was assessed with DDQ (18); statistics are given in TABLE TWO. Figures were generated using PYMOL (19).

Y2H Assays—CTDF homodimerization was probed using the MATCHMAKER Gal4 system (BD Biosciences Clontech, Palo Alto, CA). Substitutions were introduced into plasmids by PCR mutagenesis (20) and verified by DNA sequencing. pGADT7- and pGBK7-CTDF variants (residues 350–427) were co-transformed into pairs into yeast strain Y187 by the lithium acetate/polyethylene glycol method. Cells were plated on SD/−Leu/−Trp minimal medium supplemented with 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Expression levels were verified by Western blot using anti-Gal4 antibodies (Upstate Group, Charlottesville, VA). β-Galactosidase activity in colonies was monitored on X-Gal indicator plates and measured in cell extracts by o-nitrophenyl-β-D-galactoside assay.

RESULTS

Overview of Structure—The crystal structure of the CTDF-p (residues 350–412) was determined at a resolution of 1.6 Å. Representative electron density is shown in Fig. 2A. Values of R and Rfree (20.8 and 25.7%, respectively) in part reflect disorder of the N- and C-terminal segments.6 The N-terminal five residues of CTDF-p (including two residues derived from thrombin cleavage of the fusion protein) are omitted from the model. The structure exhibits good stereochemistry, with 94% of non-Gly, non-Pro residues occupying the most favored region of the Ramachandran plot and the remaining 6% occupying the additionally allowed region. No residues are observed in generously allowed or disallowed regions.

The structure of CTDF-p forms an ellipsoidal dimer with protomers oriented head to tail (Fig. 2B). Each protomer contains three α-helices and exhibits a UBA fold. Residues 350–352 are not well ordered. Helical segments are 353–367 (α1), 371–384 (α2), and 388–407 (α3). The second helix exhibits a kink at Pro-375 (red in Fig. 3), separating subsegments α2A (371–374) and α2B (red cylinder); α2A forms a 3i5i helical turn (green cylinder in Fig. 4A). Residues 384–387 comprise a type I’ β-turn; the turn at 367–371 is non-canonical. Substantial hydrophobic contacts, characteristic of a UBA fold, occur between α1 and α2 and in part by α3 (see residues Leu-363, Met-377, and Ile-395 in Fig. 2B and Fig. 5, A and B). The surface of α3 contains (i, i+3) and (i, i+4) salt bridges from Glu-390 to Arg-393 and Arg-394 (Fig. 4E). The female-specific sequence begins midway through α3 (398–407); residues 408–412 are disordered. Despite limited sequence homology (Fig. 4A and supplemental Fig. S4), structural alignment of a CTDF-p protomer with 18 UBA domains yields a mean pairwise root mean square deviation of 1.7 Å (range 1.2–2.3 Å) among main-chain atoms (Fig. 4B); the mean base-line pairwise root mean square deviation among these 18 domains is 1.3 Å. The root mean square deviation between CTDF-p and a related CUE domain (green in Fig. 4B; Ref. 21) is 1.8 Å.

Each protomer contains a small hydrophobic core centered about the side chains of Met-377 and Cys-360 (Fig. 5A). Although this “mini-core” contains residues from each of the three helices, the predominant contribution is from α2. The side chains of Met-374, Met-377, and Leu-381 (α2) project opposite Cys-360, Leu-363, and Leu-364 (α1) and also interact with Ala-391 and Ile-395 (α3). Additional contacts occur between α1 and α3 as Leu-363 packs against Ile-388, Ala-391, and Ile-395 (α3). One side of the mini-core is sealed in part by interactions Lys-366—Ser-392 and Lys-366—Glu-389 (Fig. 5A). The female-specific sequence begins midway through α3 (398–407); residues 408–412 are disordered. Despite limited sequence homology (Fig. 4A and supplemental Fig. S4), structural alignment of a CTDF-p protomer with 18 UBA domains yields a mean pairwise root mean square deviation of 1.7 Å (range 1.2–2.3 Å) among main-chain atoms (Fig. 4B); the mean base-line pairwise root mean square deviation among these 18 domains is 1.3 Å. The root mean square deviation between CTDF-p and a related CUE domain (green in Fig. 4B; Ref. 21) is 1.8 Å.

The N- and C-terminal five residues of each protomer exhibit neither complete disorder nor two or more well defined conformations, resulting in uninterpretable positive Fo−Fo density. Refinement statistics are limited by either omission of these residues from the model or their inclusion in an approximate model with high thermal factors. NMR studies provide evidence for corresponding segmental disorder in solution (J. R. Bayrer, Y. Yang, and M. A. Weiss, unpublished results).

### TABLE ONE

Data collection statistics

|                | Native                | Selenomethionine         |
|----------------|-----------------------|--------------------------|
| Wavelength (Å) | 0.9000                | 0.9788                   |
| Unit cell parameters (Å) | a = 39.8 b = 46.6 c = 59.8 | a = 39.4 b = 46.8 c = 58.6 |
| Resolution range (Å) | 19.1-6 (1.66-1.60) | 32.7-1.8 (1.86-1.80) |
| No. measured reflections | 164,648 | 145,768 |
| No. unique reflections | 15,159 (1,467) | 19,414 (1,905) |
| Redundancy | 10.9 | 7.5 |
| Completeness (%) | 99.1 (96.9) | 100 (100) |
| Rmerge (%) | 3.8 (0.182) | 8.7 (0.456) |
| Average I/s(I) | 62.3 (11) | 24.0 (3.4) |
| Correlation coefficient | 0.59 |  |
packing between &omicron;2 and &omicron;2 exhibits "knobs in holes" complementarity (Fig. 3C). 24 residues in each protomer define an extensive interface; the core is non-polar (Leu-357, Tyr-369, Trp-371, Leu-373, Met-374, Pro-375, Leu-376, Tyr-378, Val-379, Ile-395, Gly-398, Val-401, and Val-402; supplemental Fig. S2). The overall interface juxtaposes concave and convex surfaces. The contact area of a protomer (1077 Å²) spans 29% of its total surface (green regions in Fig. 7, A and B); dimerization, thus, buries a combined surface of 2153 Å². Hydrophobic packing is extended by intermolecular salt bridges, hydrogen bonds, and a bridging network of water molecules (Asp-354, Tyr-378, Lys-382, Asp-383, Arg-394, Glu-397, Gln-399, and Tyr-405; supplemental Fig. S2). Arg-394 participates in both intramolecular and intermolecular salt bridges (Glu-390 and Asp-383 (primed residues numbers refer to dimer-related protomer); Fig. 6A); Glu-397 interacts with Tyr-378 and Lys-382 (Fig. 6B). Such interactions (and neighboring solvation) apparently offset the proximity of Asp-383 and Asp-383. Minor 1-3 intermolecular interactions are also observed (involving Asp-354, Leu-357, and Tyr-398). The junction between sex-nonspecific and sex-specific portions of CTDF-p (G398) packs against &omicron;2 across the dimer interface. This junction is a site of mutation (G398D) associated with intersexual development (7). The overall structure is comprised predominantly of non-sex-specific residues and so is likely to be similar in CTDM.

Ala Scanning Mutagenesis—Y2H studies of variant domains were undertaken to test the contribution of interfacial side chains to dimerization (TABLE THREE). Bait and prey plasmids were constructed using the Gal4 system (Fig. 8A); photomicrographs of representative X-gal indicator plates demonstrate a range of -galactosidase activities (white, light blue, and blue colonies; Fig. 8, B and C). A negative control was provided by G398D (7). Colorimetric phenotypes of Y2H yeast colonies were in each case verified in cell extracts by quantitative -galactosidase assays.

17 Ala substitutions were tested; 3 substitutions in the core (Tyr-378, Ile-380, Ile-395) markedly impair -galactosidase activity whereas 3 substitutions at the surface (W371A, Y400A, and N403A) yield no perturbations. Ala substitutions at three interfacial valines in &omicron;2 and &omicron;3 exhibit partial (Val-379 and Val-402) or full (Val-401) activity; the resulting packing defects may be offset by the higher helical propensity of Ala. R394A (expected to disrupt inter- and intramolecular salt bridges) and K382A (expected to disrupt a dimer-specific charge-stabilized hydrogen bond) markedly impair activity. E396A and E397A are partially tolerated, whereas D383A has no effect. Interestingly, the two prolines, although not features of canonical UBA or CUE domains, appear essential. G398A enhances -galactosidase activity, presumably by stabilizing &omicron;3.

Putative Ub Binding Surface—A putative Ub binding surface (inferred based on known monomeric UBA and CUE domains; gold surface in Fig. 7A) does not overlap with the dimer interface of CTD-p (green surface). It is, thus, possible that the CTD-p dimer presents two Ub-binding sites, one on each side. Heteronuclear NMR titration experiments suggest weak binding of the domain to Ub at protein concentrations >100 μM (supple-
ment Fig. S1). Alignment of one protomer with CUE2 as bound to Ub (21) yields a model of a complex between Ub and the CTD\(^{2-}\)-p dimer (Fig. 4C). This model suggests that potential UBA-Ub salt bridges are conserved: Asp-18 and Asp-40 of CUE2 align with Glu-365 and Glu-389 of CTD\(^{2-}\)-p. The model also permits a second Ub molecule to bind to the other protomer. We, thus, envisage that such a dimeric UBA fold could enable bidentate recognition of a poly-Ub chain.

**DISCUSSION**

The CTDs of DSX enhance DNA recognition by providing a strong dimer contact (7, 10, 22). Although the monomeric DM domain itself can bind DNA sites as a cooperative dimer (4, 23), the CTDs enhance specific DNA binding by 35-fold (9). The biological importance of CTD dimerization is suggested by a mutation in CTD\(^{2-}\) (G398D) that blocks dimerization in association with intersexual development (7). Unexpectedly, the crystal structure of CTD\(^{2-}\)-p demonstrates that dimerization is mediated by a UBA-domain fold, previously unrecognized due to the absence of detectable sequence homology. The stability of the DSX\(^{2-}\) dimer (\(K_D \approx 10^{-10}\) M; Ref. 9) reflects formation of an extensive hydrophobic interface flanked by intersubunit salt bridges and hydrogen bonds. The intersexual mutation G398D would insert an uncompensated charge into this interface. The CTD dimer, thus, extends to the realm of transcription the repertoire of UBA dimerization, previously implicated in cell-cycle
Yeast two-hybrid analyses

Substitutions were tested in the context of fragment 350–427; deletion of residues 413–427 does not affect activity (line 2).

| Variant       | SA | Y2H |
|---------------|----|-----|
| Wild type     |    | +   |
| Δ413−427      |    | +   |
| G398D         | 0.00 | −   |
| P370A         | 0.50 | −   |
| W371A         | 0.42 | +   |
| P375A         | 0.06 | −   |
| Y378A         | 0.06 | −   |
| V379A         | 0.00 | +/− |
| I380A         | 0.00 | −   |
| K382A         | 0.51 | −   |
| D383A         | 0.04 | +   |
| R394A         | 0.12 | −   |
| I395A         | 0.00 | −   |
| E396A         | 0.59 | +/− |
| E397A         | 0.26 | +/− |
| G398A         | 0.00 | +   |
| Y400A         | 0.68 | +   |
| V401A         | 0.09 | +   |
| V402A         | 0.05 | +/− |
| N403A         | 0.79 | +   |

*SA, solvent accessibility based on average of protomers.

Control mutation associated in vivo with intersexual development of XX flies and previously shown to block Y2H dimerization (7).
control (24) and receptor trafficking (25). To our knowledge, this is the first description of the structure of a dimeric UBA fold. As discussed in turn below, this structure represents a novel class of helical dimerization domains, rationalizes results of mutagenesis, and suggests a possible role for Ub in sex determination.

Small α-helical dimerization elements are often observed in transcription factors, defining regulatory families (26). Examples include the leucine zipper (Fig. 9A; Ref. 27), the basic-region helix-loop-helix motif (Fig. 9B; Ref. 28), and the canonical four-helix bundle as exemplified by Lac repressor (Fig. 9C; Ref. 29). These structures share with CTDF-p the use of parallel α-helices and extensive interhelical contacts. In many (but not all) cases such modular motifs can mediate both homo- and heterodimerization and so enable combinatorial gene regulation (30).

Although the prototypical bZIP transcription factor GCN4 forms only homodimers, for example, mammalian homologs (including proto-oncoproteins Fos and Jun) form networks of interacting proteins. UBA domains can likewise form homo- and heterodimers (24, 31). Although DSX itself forms stable homodimers and is not known to form heterodimers with homologous DNA-binding proteins, it would be of interest should a family of UBA-containing transcription factors in Droso-

**FIGURE 8. Alanine scanning mutagenesis.** A, plasmid constructs employed in Y2H system. B and C, analysis of homodimerization by CTDF-p variants. X-gal indicator plates; blue, strong dimerization; light blue, weak dimerization; white, undetectable dimerization. AD, activation domain. DBD, DNA binding domain. wt, wild type.

**FIGURE 9. Helical dimerization motifs in transcription factors.** A, coiled-coil domain of GCN4 (PDB code 2DGC). B, helix-loop-helix domain of MyoD (PDB code 1MDY). C, four-helix bundle of Lac repressor (PDB code 1JWL). In each panel an overview of the protein-DNA complex is shown at the left (black and gray); right, stereo view showing one protomer in blue and the other in magenta.

7 It is not known whether the structural mechanisms of such UBA homo- and heterodimerization resemble that of CTDF-p.

8 The present structure is unrelated to the low affinity CUE dimer (K_d 1 mM) formed by domain swapping (supplemental Fig. S2; Ref. 41).
related factors can be repressed through formation of an inactive UBA-mediated heterodimer. Proof of principle is provided by molecular genetic studies of transgenic flies in which non-native DSXF-DSXM heterodimers with altered gene regulatory properties are envisaged (32).

The present structure rationalizes the results of alanine-scanning mutagenesis (TABLE THREE), indicating that dimerization requires steric complementarity at a hydrophobic interface and is further stabilized by flanking salt bridges. Similar features stabilize the coiled-coil structure of leucine zippers (27, 33). Our results are also in accord with previous Y2H studies in which error-prone PCR was employed to identify residues critical for dimerization (7). Although in that study the variant domains each contained two or three mutations (thus making uncertain the effect of any single substitution), the structural environment of these mutations are of interest. For L373Q and M377K, Leu-373 and Leu-373’ pack against each other to seal one edge of the dimer interface (Fig. 3C). Met-377 contributes to the mini-core of the protomer and an edge of the dimer interface (Fig. 3B). For L381S, K382R, and Q313K, whereas Gln-313 is outside of the dimerization domain, Leu-381 packs with the protomer (Fig. 5, A and B); Lys-382 forms a dimer-related charge-stabilized hydrogen bond with Glu-397’ (Fig. 6). Scanning substitution K382A in our hands likewise results in a loss of dimerization. For I395N, E418G, and R420P, Ile-395 packs within the mini-core of the protomer and at one edge of the dimer. Residues Glu-418 and Arg-420, not included in the crystallized fragment, are presumably disordered. It would be of future interest to extend this approach by random cassette mutagenesis; analysis of allowed and disallowed families of substitutions would further define the sequence requirements of UBA dimerization.

The presence of a UBA-like domain in DSX may represent the incidental recruitment of a common structural motif or indicate a functional role for ubiquitin (e.g. binding of mono- or polyubiquitinated proteins, engagement of the enzymatic machinery of ubiquitination and/or the proteasome) in DSX-mediated transcriptional regulation. Although no biological data are available, the striking structural similarity between CTD-p and the classical UBA domain suggests a potential role for the ubiquitin system in sex-specific gene regulation. As demonstrated by Lipkowitz and co-workers (31) in studies of c-Cbl and Cbl-b, the sequence of a UBA-like domain does not predict whether or not it binds Ub. Although c-Cbl and Cbl-b share 85% sequence similarity, for example, only Cbl-b is able to bind Ub in vitro. Furthermore, the Cbl-b UBA domain contains two polar residues within the otherwise hydrophobic Ub binding patch characterized among Rad23-derived UBA domains. Consideration of the potential role of Ub binding by CTD-p is, thus, tempered by the marked variability of UBA-like sequences and their heterogeneous biological functions. Indeed, although evidence for low affinity binding of Ub to CTD-p is provided by preliminary NMR titration experiments (supplemental Fig. S1), the physiological significance of such binding remains unclear.

To our knowledge, the participation of the ubiquitination machinery in the Drosophila sex-determining hierarchy has not been previously suggested. Such involvement would nevertheless be plausible in light of growing evidence that ubiquitination and ubiquitinated proteins can play central roles in the regulation of eukaryotic gene expression. (i) Ub-triggered proteolysis can control transcription through “suicidal” regulation wherein each cycle of transcription is coupled to destruction of a specific transcription factor (34). (ii) Ubiquitination can regulate subcellular localization (35) and protein-protein interactions (36). (iii) Transcriptional initiation and functional mRNA processing can require degrons, proteolytic signaling elements within transcriptional activation domains that recruit Ub ligases (37). (iv) Non-destructive ubiquitination of activation domains can enable transcriptional activation (34). It is possible that one or more of these general processes operate in transcriptional regulation by the DSX isoforms but were missed in clas-

FIGURE 10. Surface topography of CTD-p and proposed role of Ub binding in gene regulation. A and B, comparison of the putative Ub binding surface of CTD-p with the corresponding surface of a ubiquitin-associated CUE domain. A, left, surface representation of CUE2 with its well characterized Ub binding surface (21) highlighted in purple. A, right, calculated electrostatic surface. B, left, calculated electrostatic surface of CTD-p dimer. One protomer is shown in gray, and the other is in black. The putative Ub binding surface is shown in purple. B, right, calculated electrostatic surface of CTD-p. Surfaces were calculated using APBS (43). C, model of sex-specific regulation of yolk protein expression in female (top) and male (bottom; Refs. 39 and 40). Female- and male-specific C-terminal extensions of DSX are shown in red and purple, respectively. DSX binds to DNA as a dimer (green spheres, DM domain; blue and teal, CTD-p). Top, female-specific complex of DSX occupies dsx as an adjoining bZIP factor binds to bZIP1 (light blue ribbon). Recruitment of IX (arrow) enables synergistic activation of transcription. Binding of DSX or DSXM displaces AEF1 from its target site aef1. In females, fat-body expression of DSX is higher than that of AEF1, and therefore, in the presence of bZIP1, yolk proteins are expressed. Expression is by contrast repressed in ovary due to higher levels of AEF1, which displaces DSXM. Bottom, male-specific repression of yolk proteins occurs as binding of DSXM, which is 122 residues longer than DSX (purple tail; Ref. 2), occludes bZIP1 or inactivates bound bZIP1 (44). Also pictured is non-tissue-specific activator Ref1. D, schematic models of sex-specific recruitment of IX (orange rectangles) by DSX. A cylinder model of CTD-p dimer is shown in blue and teal. Weak DSX-IX interaction (top) would be strengthened by bidentate recognition of tethered Mobieties (bottom, pink triangles), providing a mechanism of ubiquitination-coupled assembly of a preinitiation complex.
tical genetic screens for impaired sexual differentiation due to the pleiotropic functions of the ubiquitination machinery.

Biochemical mechanisms of sex-specific gene regulation are not well characterized in *Drosophila*, and therefore, the relevance of Ub-related processes to DSX function cannot presently be evaluated. It is nonetheless intriguing that the topography and electrostatic potential surface of CTD–p resemble those of the Ub binding surface of the CUE domain (Fig. 10). Given the stringent conservation of this surface among DSX homologs, we propose that the DSX UBA domain may contribute to the assembly of a specific preinitiation complex through non-covalent interactions with mono- or polyubiquitinated proteins. Such an interacting protein(s) is presently unidentified. One possibility is suggested by a model based on the possible ubiquitination of DSX-associated transcriptional coactivator Intersex (IX; Ref. 8), the homolog of a mammalian Mediator subunit (38). A framework is provided by the Wensink model of sex- and tissue-specific expression of *yolk protein* genes in the fat body (Fig. 10C; Refs. 39 and 40). In this model DSX and DSXM occupy the same sites in the fat body enhancer; distinct protein-protein interactions lead to opposing gene-regulating functions (upper and lower panels of Fig. 10C). A key (and newly recognized) component is provided by IX (8): its sex-specific binding to DSXF (and so presumably to CTD–p) is required for female differentiation (32). In Y2H studies IX-DSX binding is detectable but weak. The strength and avidity of such binding could be enhanced by dual recognition of IX and a tethered Ub molecule or Ub chain (Fig. 10D).

In the future the molecular functions of the DSX isoforms in *Drosophila* development may be addressed by structure-based molecular genetics. A critical test of whether DSX functions in vivo as a Ub-binding protein, for example, could be provided by targeted mutations in its putative Ub binding surface that impairs Ub binding but not dimerization or binding of unmodified IX. Should such mutations be obtained in vitro, they might provide in vivo probes for the general involvement of Ub-dependent regulatory mechanisms (such as but not limited to bidentate IX recruitment; Fig. 10D). Of particular importance would be the developmental phenotype of mutant flies homozygous for a corresponding variant dsx allele. Possible impairment of somatic sexual differentiation would be of broad interest as a model for a UBA-associated genetic disease. Deciphering the role of the transcription-associated ubiquitination machinery in developmental decisions represents an important general challenge. Should the UBA-like domains of DSX indeed function as Ub binding motifs, studies of sex-specific gene regulation in *Drosophila* would provide an opportunity to define the biochemical contribution of ubiquitination to the operation of a metazoan genetic switch.

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REFERENCES

1. Cline, T. W., and Meyer, B. J. (1996) *Annu. Rev. Genet.* 30, 637–702
2. Burts, K. C., and Baker, B. S. (1989) *Cell* 56, 997–1010

An example of such a disease in humans is Paget’s disease of bone, commonly caused by mutations in a monomeric ubiquitin domain (46).