Structural Insight into Caenorhabditis elegans Sex-determining Protein FEM-2**

Yi Zhang‡, Haifeng Zhao‡, Jia Wang‡, Jingpeng Ge‡, Yang Li‡, Jinke Gu‡, Peng Li‡, Yue Feng‡§, and Maojun Yang††

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From the ‡Ministry of Education (MOE) Key Laboratory of Protein Sciences, Tsinghua-Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084 and the ††College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China

Background: FEM-2 is central to the sex determination pathway in C. elegans.

Results: The N-terminal domain (NTD) of FEM-2 binds to FEM-1 and FEM-3, but does not directly regulate the phosphatase activity of FEM-2.

Conclusion: The FEM-2 NTD functions as a scaffold in sex determination.

Significance: This work reveals how the novel folded NTD facilitates the role of FEM-2 in sex determination.

In the nematode Caenorhabditis elegans, fem-1, fem-2, and fem-3 play crucial roles in male sexual development. Among these three genes, fem-2 encodes a PP2C (serine/threonine phosphatase type 2C)-like protein, whose activity promotes the development of masculinity. Different from the canonical PP2Cs, FEM-2 consists of an additional N-terminal domain (NTD) apart from its C-terminal catalytic domain. Interestingly, genetic studies have indicated indispensable roles for both of these two domains of FEM-2 in promoting male development, but the underlying mechanism remains unknown. In the present study, we solved the crystal structure of full-length FEM-2, which revealed a novel structural fold formed by its NTD. Structural and functional analyses demonstrated that the NTD did not directly regulate the in vitro dephosphorylation activity of FEM-2, but instead functioned as a scaffold domain in the assembly of the FEM-1/2/3 complex, the executioner in the final step of the sex determination pathway. Biochemical studies further identified the regions in the NTD involved in FEM-1 and FEM-3 interactions. Our results not only identified a novel fold formed by the extra domain of a noncanonical PP2C enzyme, but also provided important insights into the molecular mechanism of how the NTD works in mediating the sex-determining role of FEM-1/2/3 complex.

The nematode Caenorhabditis elegans develops either as a male or as a self-fertile hermaphrodite depending on the ratio of X chromosomes to autosomes, which is also known as the X/A ratio (1). Extensive genetic analysis has established a cascade of masculinizing and feminizing activities in somatic sex determination (see Fig. 1A). Despite recent progress in the field, the molecular mechanism that regulates sex determination remains to be elucidated. The product of her-1 (HER-1) is a secreted protein that negatively regulates the transmembrane protein TRA-2A (encoded by tra-2), probably by causing its conformational change (1–4). The C-terminal region of an active TRA-2A could bind to FEM-3, thus inhibiting the active FEM complex comprising FEM-1, -2, and -3. Regarding the final step, TRA-1, which acts as a transcription repressor of male-specific genes in somatic cells, is regulated by proteasomal degradation executed by a CUL-2-based ubiquitin ligase with FEM-1 as the substrate recognition subunit and FEM-2 and FEM-3 as cofactors (5).

The three fem genes play a central role in the entire sex determination pathway. They are required for the male cell fates not only in soma and germline of XO males but also during the spermatogenesis period in XX hermaphrodites (6). Homozygous mutant alleles of these genes could cause feminization of the animal (7). For instance, animals homozygous for a null allele of fem-2(e2105) all develop as females at 25 °C irrespective of their karyotype (8). FEM-2 protein contains a C-terminal serine/threonine phosphatase type 2C (PP2C)2-like domain and exhibits dephosphorylation activity in vitro (2, 7), indicating that phosphorylation and/or dephosphorylation might act as a control mechanism in C. elegans sex determination. Indeed, mutations that abolish the phosphatase activity of FEM-2 severely reduced their rescue effects in fem-2(e2105) nematodes (2), although the substrate(s) for FEM-2 remains unknown. Distinct from classical PP2Cs, FEM-2 harbors an additional large N-terminal domain with no sequence similarity to any other domains known to date (9). Interestingly, fem-2 mutants with an N-terminal deletion also failed to rescue the phenotype of fem-2(e2105) animals (6), suggesting an indispensable role for this domain in sex determination. However, how the N-terminal domain of FEM-2 participates in regulating the sexual development in C. elegans has been unclear.

Here, we report the crystal structure of FEM-2 and reveal that although the C-terminal domain of FEM-2 has a similar

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The atomic coordinates and structure factors (code 4JND) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The full-length, NTD (residues 1–160), CTD (residues 161–449), and ΔN41 (residues 42–449) of *C. elegans* FEM-2 cDNA were all cloned into pGEX6p-1 vector to produce GST-tagged fusion protein with a PreScission protease cleavage site between GST and the target protein. The FEM-2 mutants were generated by two-step PCR and were subcloned, overexpressed, and purified in the same way as wild-type protein. The target protein was expressed in *Escherichia coli* strain BL21 and induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside when the cell density reached an A_{600 nm} of 0.8. After growth at 23 °C for 7 h, the cells were harvested, resuspended in lysis buffer (1× PBS, 2 mM DTT, and 1 mM PMSF), and lysed by sonication. The cell lysate was centrifuged at 20,000 × g for 45 min at 4 °C to remove cell debris. The supernatant was applied onto a self-packaged GST affinity column (2 ml glutathione-Sepharose 4B; GE Healthcare), and contaminants were removed with wash buffer (lysis buffer plus 200 mM NaCl). The fusion protein was then digested with PreScission protease at 4 °C overnight. The protein with an additional five-amino acid tag (GPLGS) at the N terminus was eluted with lysis buffer. The eluant was concentrated using an Amicon filter unit (Millipore) and further purified using a Superdex-200 (GE Healthcare) column equilibrated with a buffer containing 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 1 mM DTT. The purified protein was analyzed by SDS-PAGE. The fractions containing the target protein were pooled and concentrated to 20 mg/ml.

The fragment of *C. elegans* Ca^{2+}/calmodulin-dependent protein kinase II (cCaMKII) cDNA (residues 1–343) was cloned into pET28a vector to produce a His-tagged fusion protein. The fusion protein was induced in *E. coli* Rosetta (DE3) by 0.2 mM isopropyl-β-D-thiogalactopyranoside when the cell density reached an A_{600 nm} of 0.8. Recombinant His-tagged protein was purified by nickel affinity column chromatography and ion exchange chromatography and was further subjected to gel filtration chromatography (Superdex-200 column) in buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM DTT.

**Crystallization**—The full-length FEM-2 protein was concentrated to 20 mg/ml in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM DTT. Crystals were grown using the hanging-drop vapor diffusion method. Crystals of FEM-2 were grown at 18 °C by mixing an equal volume of the protein (20 mg/ml) with reservoir solution containing 28% PEG400, 0.2 mM MgCl_2, 0.1 mM HEPES sodium, pH 7.4.

**Data Collection and Structural Determination**—FEM-2 crystals were soaked into 200 mM NaI for 30 s and flash-frozen in the cold nitrogen stream. Single-wavelength anomalous dispersion data were collected at 100 K using a MARResearch M165 CCD detector at the Beijing Synchrotron Radiation Facility. FEM-2 native data were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility (SSRF). All diffraction data were processed with HKL2000 (10). Further processing was carried out using programs from the CCP4 suite (Collaborative Computational Project) (27). CC1/2 was used to determine the high resolution limit of the diffraction data (11). The heavy atom positions in the iodine-soaked mutant crystal were determined using SHELDX (12). Heavy atom parameters were then refined, and initial phases were generated in the program PHASER (13) with the single-wavelength anomalous dispersion experimental phasing module. The real-space constraints were applied to the electron density map in DM (14). The resulting map was of sufficient quality for model building of the FEM-2 molecules in COOT (15). The structures were refined with the PHENIX (16) packages. Full data collection and structure statistics are summarized in Table 1.

**Autophosphorylation of cCaMKII**—The purified cCaMKII protein (10 mg/ml) was autophosphorylated at 4 °C for 1 h in the reaction mixture containing 25 mM Tris, pH 7.5, 1 mM CaCl_2, 10 mM MgCl_2, 4 mM ATP, 0.1 mM EDTA, and 0.1 mM calmodulin. After the reaction, the mix was applied to ion exchange chromatography followed by a gel filtration chromatography (Superdex-200 column) using buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM DTT on the FPLC system (GE Healthcare Life Sciences). The target protein was concentrated and stored at −80 °C.

**Enzyme Assays for FEM-2 and Its Mutants**—The phosphorylation states of cCaMKII in the absence and presence of FEM-2 were assessed by Western blot analysis using the antibody against phospho-Thr (Cell Signaling Technology). Dephosphorylation of the phosphorylated cCaMKII was determined using a continuous spectrophotometric assay (17, 18). This assay incorporates a coupled enzyme system, which uses purine nucleoside phosphorylase and its chromogenic substrate 7-methyl-6-thioguanosine for the quantification of inorganic phosphate produced in the phosphatase reaction (18). All experiments were carried out at 25 °C in 1.6 ml of reaction mixture containing 100 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM MnCl_2, 100 μM 7-methyl-6-thioguanosine, and 0.1 mg/ml purine nucleoside phosphorylase. The reactions were initiated by the addition of the phosphatase. The time courses of absorbance change at 360 nm were recorded on a Lambda 45 spectrophotometer (PerkinElmer Life Sciences) equipped with a magnetic stirrer in the cuvette holder. Initial rates were determined from the linear slope of progress curves obtained. Quantitation of phosphate release was determined using the extinction coefficient of 11,200 M⁻¹cm⁻¹ for the phosphate-dependent reaction at 360 nm (19). The concentration of 7-methyl-6-thioguanosine was determined at 331 nm, using a molar extinction coefficient of 32,000 M⁻¹cm⁻¹.
Crystal Structure of FEM-2

Eukaryotic Expression Constructs—cDNA sequences for wild-type and mutated forms of FEM-2 and its NTD and CTD were all cloned into the pCMV5-FLAG vector. FEM-1 and FEM-3 cDNA sequences were cloned into pCMV-HA and pCMV-Myc vector, respectively.

Co-immunoprecipitation Experiments—HEK293T cells cultured in 60-mm dishes were transfected with the indicated plasmids. Cells were lysed in 800 μl of cell lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 mM MgCl2, 0.5% Nonidet P-40, 10% glycerol, 1 mM DTT, 0.1 mM Na3VO4,1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and sonicated at 4°C for 30 s at level 2 (Ultrasonic Processor, Sonics). Immunoprecipitation was done in 750 μl of whole-cell lysates incubated with 2 μg of indicated antibody and 30 μl of protein G-Sepharose beads (Santa Cruz Biotechnology) at 4°C for 8 h. The beads were washed four times with cell lysis buffer, and precipitates were eluted with 2×SDS-PAGE sample buffer and analyzed by Western blotting.

RESULTS

Crystal Structure of the Full-length FEM-2—To better understand the role of the N-terminal extension of FEM-2, we first determined the structure of full-length FEM-2 at 1.65 Å resolution (Table 1), which consists of residues from 13 to 436. The region spanning residues from 45 to 60 is invisible, probably due to intrinsic flexibility. Consistent with sequence analyses (7), FEM-2 comprises two domains: an N-terminal domain (residues 13–160, referred to as NTD hereafter), and a C-terminal PP2C-like domain (residues 161–436, referred to as CTD hereafter) (Fig. 1B). Similar to other members of the PP2C family (20, 21), the CTD contains a central buried β sandwich constituted by two sets of antiparallel β sheets (Fig. 1B). In FEM-2, one set of β sheets is flanked by two α helices in an antiparallel fashion, and the other set is flanked by three α helices (Fig. 1B). A large cleft is generated between the two sets of β sheets.

The interface between NTD and CTD buries a surface area of 929.9 Å2. The interactions between the two domains are distributed in two regions, mainly composed of hydrogen-bonding interactions and water-mediated interactions (Fig. 2A). In one region (Fig. 2B), the two side-chain hydroxyl groups of NTD (D71) form hydrogen bonds with the polar groups in the side chains of CTD (D215) and CTD (R252). One side-chain hydroxyl group of NTD (D67) forms two hydrogen bonds with the polar groups in the side chains of CTD (D212) and CTD (R248). Moreover, water-mediated interactions could be observed between the carbonyl group of NTD (D111) and the side chain-hydroxyl group of CTD (D219), the carbonyl group of NTD (D166) and the side-chain amino group of CTD (R248), and the side-chain polar group of NTD (D166) and the side-chain hydroxyl group of CTD (D212). In the other region (Fig. 2C), the main-chain amino group of NTD (G177) forms a hydrogen bond with the hydroxyl group of CTD (D395), and one side-chain amino group of NTD (R119) forms a hydrogen bond with the carbonyl group of CTD (I161). The side-chain amino group of CTD (D218) forms two hydrogen bonds with the hydroxyl group of NTD (D156) and the carbonyl group of NTD (D157), and the side-chain hydroxyl group of CTD (D218) forms two hydrogen bonds with the main-chain amino group and side-chain-hydroxyl group of NTD (S159). Residue Asp-88 from NTD forms water-mediated interactions with Val-163 and Tyr-397 from CTD.

The CTD of FEM-2 Is Highly Conserved—PP2Cs belong to the metal-dependent protein phosphatase (PPM) family and require manganese or magnesium ions (Mg2+/Mg2+) for their activity. In the structure, two Mg2+ ions are identified in the catalytic core, both of which are hexa-coordinated by oxygen atoms from amino acids and water molecules (Fig. 3A), consistent with the structure of human PP2Ca (21). In addition, a third Mg2+ ion is found on the surface of the protein, away from the catalytic core. This is probably due to its nonspecific binding to the protein. Notably, the residues involved in Mn2+ ions and phosphate interactions in human PP2Ca are highly conserved among FEM-2 homologs from different nematode species and Homo sapiens (Figs. 3B and 4). In the bacterial PPM homologs, three metal ions in the active site and a flap region were identified (21), which is inconsistent with the situation in the present structure, indicating FEM-2 as a characteristic eukaryotic PP2C (Fig. 5C). Importantly, although the structural features of the

| TABLE 1 |
| --- |
| **Data collection and refinement statistics**

Values in parentheses are for the highest resolution shell. I-SAD, iodine-based single-wavelength anomalous dispersion; BSRF, Beijing Synchrotron Radiation Facility; SSRF, Shanghai Synchrotron Radiation Facility.

| Data collection | Refinement |
| --- | --- |
| **Diffraction beam** | BSRF | SSRF |
| **Space group** | C222, | C222 |
| **Unit cell** | a, b, c (Å) | 82.2, 159.9, 77.2 | 81.4, 160.1, 77.4 |
| **Wavelength (Å)** | 1.542 | 0.979 |
| **Resolution (Å)** | 50.0–2.70 (2.80–2.70) | 29.8–1.65 (1.71–1.65) |
| **Rmerge** | 12.2 (66.1) | 9.1 (80.1) |
| **I/σI** | 20.77 (2.70) | 25.13 (1.5) |
| **Completeness (%)** | 100 (100) | 99.6 (98.2) |
| **Redundancy** | 12.1 (11.7) | 4.7 (3.5) |
| **Wilson B factor (Å2)** | 66.6 | 21.8 |
| **No. of reflections** | 14,334 | 60,536 |
| **SAD phased** | 61 | 61 |
| **Anomalous scatterers** | 0.34 | 0.34 |
| **Figure-of-merit (FOM)** | 0.66 | 0.66 |
| **Ramachandran plot statistics (%)** | Favorable 98.28 Allowed 1.72 Outliers 0 |
| **PDB ID** | 4JND |

Note: *Rmerge = Σ|Ih,i − Iobs|/ΣIh,i, where Iobs is the mean intensity of the observation from symmetry-related reflections of Ih.*

*Refinement using SAD phasing.*

*Values in parentheses are for the highest resolution shell.*

**Wilson B factor (Å2)**

**Side chain** 29.7

**Main chain** 23.1

**Water** 38.5

**Ligands** 22.3

**r.m.s.d.** bond lengths 0.0065

**r.m.s.d.** bond angles 1.0376

**Ramachandran plot statistics (%)**

**Favored** 98.28

**Allowed** 1.72

**Outliers** 0

**PDB ID** 4JND

**Data set**

**Unit cell** 82.2, 159.9, 77.2

**Wavelength (Å)** 1.542

**Resolution (Å)** 50.0–2.70 (2.80–2.70)

**Rmerge** 12.2 (66.1)

**I/σI** 20.77 (2.70)

**Completeness (%)** 100 (100)

**Redundancy** 12.1 (11.7)

**Wilson B factor (Å2)** 66.6

**No. of reflections** 14,334

**SAD phased** 61

**Anomalous scatterers** 0.34

**Figure-of-merit (FOM)** 0.66

**Ramachandran plot statistics (%)**

**Favored** 98.28

**Allowed** 1.72

**Outliers** 0

**PDB ID** 4JND
FEM-2 CTD are highly conserved among known PP2C proteins (Fig. 5), comparisons of the structure of FEM-2 CTD with those of an additional three PP2C family proteins reveal different orientations in α6 and α7 of FEM-2, the two α helices flanking the β sandwich on the side adjacent to the NTD. It might be caused by the interactions from the NTD because no additional N-terminal regions are present in the other three structures.

FIGURE 1. Overall structure of FEM-2. A, model of somatic sex determination in C. elegans (1). Feminizing factors (red) and masculinizing factors (black) are shown. Lines with bars represent negative interactions, and lines with arrows indicate positive interactions. A low X/A ratio (0.5) brings about male development by negatively regulating tra-1. A high X/A ratio (1.0) leaves tra-1 free to direct female somatic development. B, overall structure of FEM-2. The protein is shown in a graphic model, with its NTD in cyan and its CTD in light magenta. The Mg²⁺ ions are shown as spheres. The invisible region (residues from 45 to 60) is represented as a dashed line. The helices in the NTD are marked. The N and C termini are indicated. All the structure figures were prepared with PyMOL (26).

FIGURE 2. Graphic representation of the interface between NTD and CTD of FEM-2. The color scheme is the same as in Fig. 18. Residues involved in binding are indicated and shown as sticks. Hydrogen bonds are indicated as red dashed lines. Enlarged views of the parts in the black and red box in A are shown in B and C, respectively.
FIGURE 3. The NTD does not directly regulate the dephosphorylation activity of FEM-2 in vitro. A, the binding sites of Mg\textsuperscript{2+} ions. Mg\textsuperscript{2+} ions and water molecules are shown as light blue and red spheres, respectively. The residues involved in Mg\textsuperscript{2+} coordination are shown as sticks. Metal-oxygen coordination bonds are shown as red dashed lines. B, superposition of the conserved residues involved in metal coordination between FEM-2 and human PP2C\textalpha. Residues from FEM-2 and human PP2C\textalpha (indicated in parentheses) are shown as yellow and green sticks, respectively. Mg\textsuperscript{2+} ions in FEM-2 and Mn\textsuperscript{2+} ions in PP2C\textalpha are shown as light blue and light green spheres, respectively. C, dephosphorylation of cCaMKII by FEM-2 in vitro. The phosphorylation state of cCaMKII is shown in the absence and presence of FEM-2, respectively. Upper row, phospho-Thr antibody detection; lower row, Coomassie Blue staining. D, in vitro phosphatase activity assays of FEM-2 and its mutants. The assay was performed in the presence of Mn\textsuperscript{2+}, Mg\textsuperscript{2+}, and 5\textmuM of phosphorylated cCaMKII protein and wild-type FEM-2 (FEM-2 FL), its CTD (FEM-2 CTD), its NTD (FEM-2 NTD), and three mutants, respectively, all at a final concentration of 0.05\textmuM. Each assay was performed in triplicate, and standard deviations are represented as error bars.

FIGURE 4. Sequence alignment of FEM-2 homologs from C. elegans, Caenorhabditis remanei, Caenorhabditis brenneri, Caenorhabditis briggsae, and H. sapiens. Residues with 100% homology, over 75% homology, and over 50% homology are shaded in dark blue, pink, and light blue, respectively. The conserved residues involved in phosphate and Mg\textsuperscript{2+} binding are marked with stars.
The NTD Does Not Directly Regulate the Phosphatase Activity of FEM-2—The most striking feature of the FEM-2 structure lies in its NTD (Fig. 1B). A Dali search with this domain only returned entries with low Z-scores of 4.3–3.9, suggesting that no known structure was identified to share significant homology with this domain. That is, NTD of FEM-2 likely represents a novel structural fold. The helical NTD mainly comprises four α helices with kinks in α1 and α4 and a long loop connecting α3 and α4 (Fig. 1B). The electron density for the region linking α1 and α2 was invisible, possibly due to its flexibility. Secondary structural predictions indicated that this region might mostly form loops (data not shown). To determine the biological functions of this novel domain, we tested whether it could regulate the phosphatase activity of the CTD, because in many cases, the activity of a phosphatase could be regulated by the subdomain structures (22). Although no endogenous substrate has been identified for FEM-2 until now, Tan et al. (23) reported that hFEM-2, the human homolog of FEM-2, could efficiently dephosphorylate CaMKII in vitro. Thus, we carried out an in vitro phosphatase assay with the C. elegans homolog of CaMKII (cCaMKII) as a substrate. The wild-type FEM-2 showed robust phosphatase activity toward phosphorylated cCaMKII in vitro. However, when compared with the full-length FEM-2, the CTD alone showed a similar phosphatase activity at least in the in vitro assay, suggesting that the NTD does not have a direct function on the in vitro phosphatase activity of FEM-2. This is consistent with the previous finding that removal of the N-terminal domain had no effect on FEM-2 rescuing the growth defect in a yeast PP2C mutant ptc1 (6). In addition, evidence from the structure indicated that the NTD does not appear to alter the conformation of the active site in the CTD, based on the structure similarities of the CTD with other PP2C homologs (Fig. 5). Taken together, the NTD does not directly regulate the phosphatase activity of FEM-2 in vitro.

The NTD Mediates the Binding of FEM-2 to FEM-1 and FEM-3—It was reported that three FEM proteins (FEM-1, -2, and -3) could interact with each other in vitro to constitute a functional FEM-1/2/3 complex, mediating male sexual development (5). Thus, we proposed that the NTD of FEM-2 might participate in protein-protein interaction in the assembly of the FEM complex. To test that, we conducted co-immunoprecipitation in HEK293T cells to examine the binding of full-length FEM-2 and the NTD and CTD of FEM-2 to FEM-1 and FEM-3. Indeed, the NTD itself could interact with FEM-1 and FEM-3 simultaneously, with similar efficiency when compared with full-length FEM-2 (Fig. 6A). In contrast, the CTD could only bind to FEM-1, with a much lower efficiency than the full-length protein (Fig. 6B). Supporting our results, GST pulldown assay (23) revealed that mammalian PP2Ca could not bind to either FEM-1 or FEM-3, indicating that the sole PP2C domain is not sufficient for FEM-1 and FEM-3 binding. Next, we set out to identify the region(s) responsible for the interaction of FEM-2 with the other two FEM proteins. Because there is no structural information for FEM-1 and FEM-3, we focused on the conserved surface of FEM-2 NTD. Notably, α1 of the NTD (residues from 23 to 40), together with the region before it, constitutes a part of the protein surface and does not form tight interactions with the rest of the NTD (Fig. 6C). We therefore tested whether this region mediated the interactions with FEM-1 and/or FEM-3. Deletion of this N-terminal region (FEM-2 ΔN41) severely impaired the binding of FEM-2 to both FEM-1 and FEM-3 (Fig. 6, E and F). Moreover, our detailed examination identified a negatively charged patch formed by several acidic amino acid residues, which are highly conserved among FEM-2 homologs from different nematode species (Fig. 6D). To explore the functional significance of these residues, we generated a FEM-2 variant (Mut1) containing the mutations E28A/E29A/D33A/E34A and tested its binding to FEM-1 and FEM-3. This mutant showed a significantly diminished interaction with FEM-1 and FEM-3, similarly to that observed with the FEM-2 ΔN41 mutant, suggesting that the interaction of FEM-2 with FEM-1 and FEM-3 could be mainly attributed to this acidic region. To examine the role of the invisible region in the structure (residues from 45 to 60), we generated another variant of FEM-2 (Mut2) by mutating three conserved residues Ile-54/Arg-55/Phe-56 to alanines. Interestingly, although Mut2 retained interaction with FEM-1, it failed to bind to FEM-3, which indicated that this invisible region in the structure might be involved only in FEM-3 binding. Finally, we made a third FEM-2 variant with another set of three surface residues mutated, named Mut3 (D71A/H74A/D75A). However, it displayed similar binding activity to both FEM-1 and FEM-3 as wild-type FEM-2 (Fig. 6, E and F). All of these mutations did not disrupt the overall fold of FEM-2 as the mutant proteins displayed similar pro-
files as wild-type FEM-2, with the only exception that a small fraction of FEM-2 ΔN41 was in an oligomerized state (Fig. 7).

Collectively, our experiments indicated that the NTD of FEM-2 could act as a scaffold mediating the assembly of the FEM complex by binding to FEM-1 and FEM-3 simultaneously. A negatively charged patch formed by α1 in FEM-2 NTD is involved in both interactions, and the region containing residues from 45 to 60 also mediates the binding to FEM-3.

**DISCUSSION**

In the study, we report the crystal structure of *C. elegans* FEM-2, a PP2C-like enzyme with a large N-terminal noncatalytic domain. In fact, extra domain-containing PP2C-like proteins also exist in other organisms, especially in plants (22). For instance, *Arabidopsis* PP2Cs ABI1, ABI2, AtP2C-HA, AthPP2CA, and kinase-associated protein phosphatase (KAPP) all contain N-terminal extensions. To our knowledge, the struc-
FEM-1 and FEM-3. Mutagenesis analyses revealed that the fold for the assembly of the FEM complex by interacting with FEM-2 and provided evidence supporting its function as a scaffolding event happens.

This gene in the sex determination pathway. Further investigations are still needed to ascertain whether this dephosphorylation event happens in vivo and whether cCaMKII or its downstream targets might play a role in sex determination.

We identified a novel structural fold formed by the NTD of FEM-2 and provided evidence supporting its function as a scaffold for the assembly of the FEM complex by interacting with FEM-1 and FEM-3. Mutagenesis analyses revealed that the N-terminal region (mainly α1) is involved in binding to both FEM-1 and FEM-3, and such interaction can be mainly attributed to the four conserved negatively charged residues on α1 (Mut1) we identified (Fig. 6, E and F). These results suggested that the binding sites of FEM-1 and FEM-3 on FEM-2 might be overlapping. Alternatively, the negatively charged region might represent an interface where the three proteins interact with each other. In addition, the region from residue 45 to 60 of FEM-2 also contributes to FEM-3 binding, supporting that the interaction between FEM-2 and FEM-3 involves more than one binding site. Detailed information of the interaction mode still awaits the determination of the structure of FEM-1/2/3 complex. Because all three FEM proteins are required for the normal male development and the FEM complex promotes TRA-1 degradation, the interaction of FEM-2 with the other two FEM proteins therefore should be essential for male development. Further studies are warranted to investigate whether fem-2 mutants with impaired FEM-1 or FEM-3 binding ability would also interfere with their function in promoting male development.

For many extra domain-containing PP2Cs, those additional domains have been proposed to regulate the activity of the enzymes (22). Our structural and biochemical analyses demonstrate for the first time how such a domain can regulate the PP2C protein by mediating protein-protein interaction, rather than directly controlling the phosphatase activity. In the case of FEM-2, its NTD is critical in interactions with FEM-1 and FEM-3. We propose that, similar to the function of the regulatory subunits in protein phosphatase 1 (PP1) enzymes (21), the NTD of FEM-2 acts as a regulatory module to facilitate the formation of a functional phosphatase complex FEM-1/2/3, leading to the effective dephosphorylation of their substrate in vivo.

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