

Toward artificial developmental regulators

– Supporting Information –

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Abbreviations. Acetylated bovine serum albumin (BSA), *N,N*-Dimethylformamide (DMF), Dimethylsulfoxide (DMSO), *N,N*-Diisopropylethylamine (DIEA), *rac*-Dithioerythrol (DTT), 4-Hydroxy- β -cyano-cinnamic acid (4-HCCA), (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), 1-Hydroxybenzotriazole (HOBt), 1-Hydroxybenzotriazolyl-tetramethyluronium hexafluorophosphate (HBTU), Trifluoroacetic acid (TFA), Trishydroxymethylaminomethane (TRIS).

Materials. Boc- β -Ala-PAM resin (0.59 mmol/g), anhydrous HOBt and HBTU were purchased from Peptides International. SASRIN[™] resin and all Fmoc/*t*Bu protected α -amino acids were from Bachem, TFA was from Halocarbon, and DMSO was from Fisher scientific. All other solvents and reagents were anhydrous and/or ACS grade purchased from VWR or Aldrich and used as received. Water was purified using a Millipore MilliQ water purification system (18 M Ω). Biochemical experiments were performed using RNase free water (Invitrogen). DNase I and calf thymus DNA were purchased from Amersham, all other enzymes and materials for molecular biology was from Roche. All buffers were 0.2 μ m filtered before storage. Oligonucleotide oligomers were from Integrated DNA Technologies Inc.

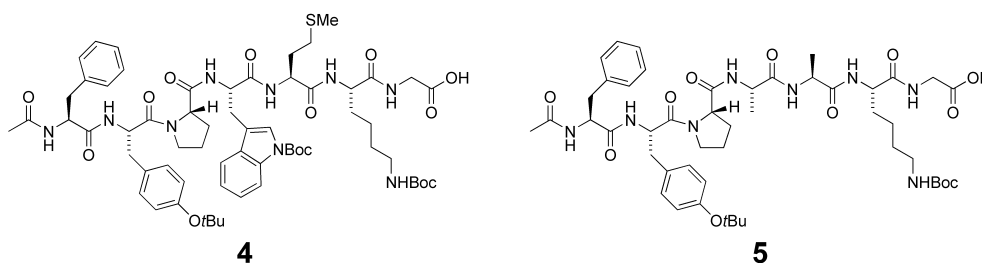
Methods. UV spectra were recorded on a HP8452A diode array spectrophotometer. All polyamide compound concentrations were determined by UV spectroscopy (H₂O) employing $\epsilon = 69500 \text{ Lmol}^{-1}\text{cm}^{-1}$ at λ_{max} near 312 nm. ESI and MALDI-TOF mass spectra were recorded at the CalTech mass spectrometry service facility on a Finnigan LC-Q (2 μ M in 50% acetonitrile, 5 μ L/min) or a Perseptive Biosystems Voyager instrument (5 pmol samples in 4-HCCA matrix).

Analytical HPLC was performed on a Beckman Gold HPLC System fitted with a diode array detector and a Varian-RP18 microsorb column (250 × 4.6 mm) at 1 mL/min, 0-100% CH₃CN in 0.1% TFA (v/v) in 30 min. Preparative HPLC was performed on a Beckman Gold HPLC System fitted with a diode array detector and a Waters DeltaPak-RP18 column (25 × 100 mm) equipped with a guard, at 8 mL/min (0-50% CH₃CN in 0.1% TFA in 50 min, *Method #1*), or a DeltaPak-RP18 column (25 × 100 mm) equipped with a guard attached to a Varian Dynamax-RP18 column (21.4 × 250 mm), at 16 mL/min (0-40% CH₃CN in 0.1% TFA in 70 min, *Method #2*).

Polyamide. Polyamide **1** was synthesized by manual solid phase synthesis following established procedures.^[1] Cleavage from PAM resin was accomplished by aminolysis with neat Dimethylaminopropylamine (37°C, 12 h). The volatiles were removed *in vacuo*, the residue taken up in 10% AcOH and purified by prep. HPLC (*Method #2*). HPLC 14.6 min. MS (ESI) [M+H]⁺ calcd for C₅₉H₇₆H₂₃O₁₀ 1266.6, found 1266.4.

Protected peptide acid synthesis. *t*Bu-protected peptide acids were synthesized by manual solid phase synthesis on SASRINTM resin.^[2] In brief, 125 mg of SASRINTM resin (1.08 mmol eq/g) were placed in a presiliconized peptide synthesis vessel, preswollen in CH₂Cl₂ (10 min), and combined with a premixed (30 min) and filtered solution of Fmoc-Gly-OH (150 mg, 0.5 mmol, 4 eq) in DMF (125 μL) and DCC (500 μL, 1.0 M in CH₂Cl₂, 0.5 mmol, 4 eq). DMAP (6 mg, 0.05 mmol, 0.1 eq) was added, and the mixture was shaken for 12 h. After draining and washing (CH₂Cl₂, DMF, CH₂Cl₂), the loaded resin was capped by treatment with benzoyl chloride/pyridine/CH₂Cl₂ 1:1:3 (1.25 mL) for 30 min. Fmoc deprotection was in general achieved by treatment with 25% piperidine in DMF (3 ×; 2 sec, 30 sec, 15 min), but the second residue was deprotected with 50% piperidine in DMF (3 ×; 2 sec, 30 sec, 5 min). Amino acid coupling was performed for 1.5 h at r.t. using a solution of 0.3 mmol Fmoc/*t*Bu protected amino acid in DMF (0.7 mL) preactivated with 0.3 mmol HOBt, 0.27 mmol HBTU and 50 μL of DIEA for 5 min. After 15 min of coupling time, more DIEA (20 μL) was added to the mixture. After successive build-up of the peptide chain, the terminal Fmoc group was removed and the resin bound peptide treated with a mixture of Ac₂O/pyridine/DMF 2:3:10 (1 mL) for 30 min followed by thorough washing (DMF, *i*PrOH, DMF, CH₂Cl₂). The peptide was cleaved from the resin in four cycles, where the resin was treated with TFA/Ethanedithiol/Et₃SiH/CH₂Cl₂ 1:5:5:89 (1.5 mL) for 15 min. After each cycle, the resin was drained, and the obtained solution was

immediately cooled to 0°C and neutralized with pyridine (20 μ L). All cleavage solutions were combined and partitioned between EtOAc (70 mL) and 0.1 M KHSO₄ (30 mL). The organic layer was washed with brine (2 \times 20 mL), dried with Na₂SO₄, and the volatiles were evaporated. Purification of the residue by flash column chromatography (20 g of silica) yielded the pure peptide acids. **Peptide 4**: Yield 148.5 mg (121 μ mol, 88%); TLC (CH₂Cl₂/MeOH/HCOOH 100:5:1) R_f = 0.16; HPLC 23.4 min; MS (ESI, neg.) [M-H]⁻ calcd for C₆₃H₈₆N₉O₁₄S 1224.6, found 1224.5. **Peptide 5**: Yield 54.2 mg (57 μ mol, 42%); TLC (CH₂Cl₂/MeOH/HCOOH 100:10:1) R_f = 0.19; HPLC 18.0 min; MS (ESI, neg.) [M-H]⁻ calcd for C₄₈H₆₉N₈O₁₂ 949.5, found 949.5.



Synthesis of the polyamide-peptide conjugates. A solution of 10 μ mol (4 eq.) of the respective peptide acid in CH₂Cl₂/DMF 10:1 (2.5 mL) was treated at r.t. with 0.1 M HBTU in DMF (110 μ L, 11 μ mol) and 1.0 M DIEA in DMF (12 μ L, 12 μ mol) for 5 min, before approx. 2.5 μ mol of polyamide **1** TFA salt in DMF (2.5 mL) were added, followed by 12 μ L of 1.0 M DIEA in DMF. After the conversion was complete (2h, HPLC control), the volatiles were removed *in vacuo*, and the residue was dissolved in TFA/CH₂Cl₂/Ethanedithiol/Et₃SiH 80:10:5:5 (1 mL). After 20 min, the crude peptides were precipitated with cold Et₂O (10 mL, 0°C) and isolated by centrifugation and discarding of the supernatant. The colorless powder was resuspended twice in Et₂O (5 mL, 0°C), reisolated by centrifugation, and then taken up in 0.2 M AcOH. After standing for 4 h, this solution was purified by prep. HPLC (*Method #1*) to yield the conjugates in >97.5% HPLC purity (312 nm). Conjugate **2**: Yield 3.7 mg (1.66 μ mol, 62%) from **4** and 2.67 μ mol **1**; HPLC 17.0 min; MS (MALDI-TOF) [M+H]⁺ calcd for C₁₀₈H₁₃₇N₃₂O₁₉S 2218.1, found 2218.0. Conjugate **3**: Yield 1.4 mg (0.72 μ mol, 26%) from **5** and 2.8 μ mol **1**; HPLC 15.5 min; MS (MALDI-TOF) [M+H]⁺ calcd for C₉₈H₁₂₈N₃₁O₁₉ 2043.0, found 2042.9.

DNase I footprinting. Dissociation constants for the DNA binding of compounds **1**, **2** and **3** were obtained following published protocols.^[3] All reactions were carried out in 400 μ L total volume employing 20 kcpm of a 3'-radiolabelled 250-bp restriction fragment from the plasmid

pDEH9 (Figure S1 a). No carrier DNA was used in the equilibration, and the solutions were allowed to equilibrate for 12 h at 22°C in TKMC buffer (10 mM TRIS, 10 mM KCl, 5 mM MgCl₂, 5 mM CaCl₂; pH 7.0) prior to the DNase I digestion. Reaction products (8 kcpm) were resolved on denaturing 8% polyacrylamide sequencing gels run at 55 W.

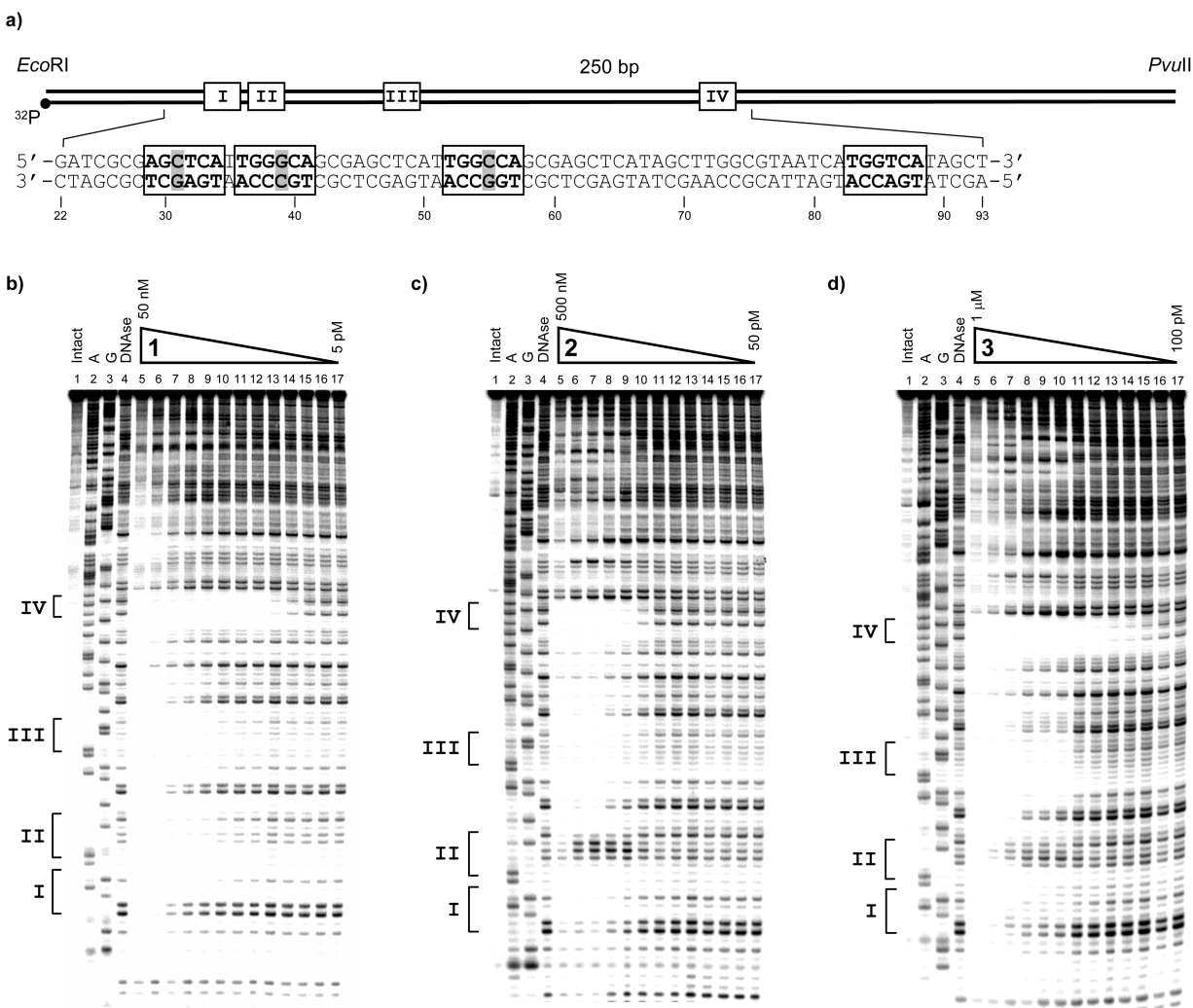


Figure S1. a) Overall composition and insert sequence of the *EcoRI/PvuII* restriction fragment from the plasmid pDEH9. Polyamide binding sites are highlighted with boxes, mismatched base pairs are shaded in gray. The site of the 3'-³²P-labeling is indicated (lower strand). b)-d) Quantitative DNase I footprint titration experiments for compounds **1**, **2** and **3** on the 3'-³²P-labeled 250-bp *EcoRI/PvuII* restriction fragment from the plasmid pDEH9. Lane 1: Intact DNA. Lane 2: A-reaction. Lane 3: G-reaction. Lane 4: DNase I standard. b) Compound **1** Lane 5-17: 50 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 500 pM, 200 pM, 100 pM, 50 pM, 20 pM, 10 pM, 5 pM; c) Compound **2** Lane 5-17: 500 nM, 200 nM, 100 nM, 50 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 500 pM, 200 pM, 100 pM, 50 pM; d) Compound **3** Lane 5-17: 1 μM, 500 nM, 200 nM, 100 nM, 50 nM, 20 nM, 10 nM, 5 nM, 2 nM, 1 nM, 500 pM, 200 pM, 100 pM, respectively. The analyzed binding site locations are indicated with square brackets along the left side of each autoradiogram.

Protein expression. *Drosophila* extradenticle (Exd) protein comprising the homeodomain and the extended fourth helix (residues 238-324),⁴ as well as ultrabithorax (Ubx) protein homeodomain (residues 233-313 of the Ubx isoform IVa) was expressed and purified after Passner and Aggarwal.⁵ The purified proteins Exd and Ubx were used for EMSA studies as described below.

EMSA (gel shift) studies.

For the templates, following DNA oligos were used (see below). The DNA upper strand was annealed with the respective matching lower strand and both strands were 5'-labeled with γ -³²P-ATP and polynucleotide kinase, using standard procedures.

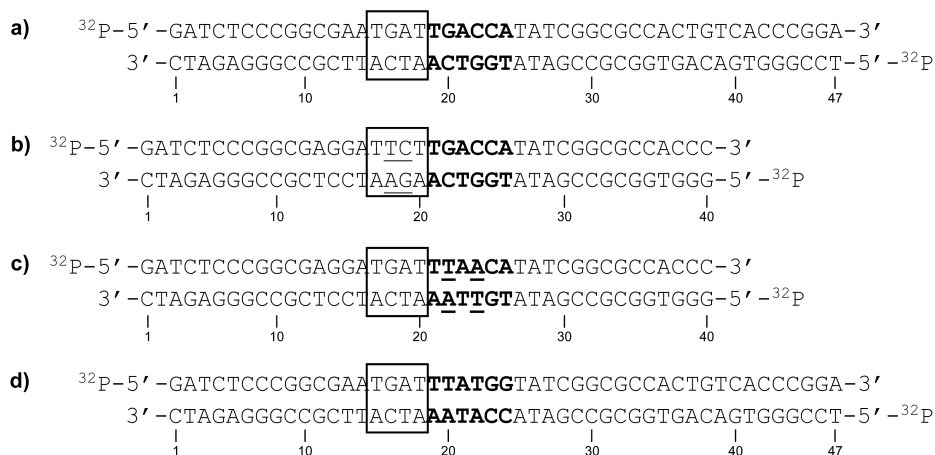


Figure S2. DNA duplexes used for the EMSA studies. The binding site for the Exd protein is marked by a box, the polyamide or Hox protein binding site is shown in boldface. a) Optimal template; b) 2-bp mismatch in the Exd site; c) 2-bp mismatch in the PA binding site; d) composite Ubx-Exd binding site (see ref. 5).

Gel-shift experiments: The master mix contained 50% BSA/50% glycerol, reaction buffer (150 mM potassium glutamate, 50 mM HEPES pH 7.0, 1 mM DTT) and 5'-end labeled DNA (³²P). The final concentrations in the samples were 100 ng/ μ L BSA and 10% glycerol. Polyamides were kept in subdued lighting whenever possible. Upon addition of the polyamide to 1 pM DNA the samples were incubated at 25°C for 30 minutes in a 20 μ L reaction. Next, Exd was added to the samples and incubated for 1 hour at 4°C. A 9% acrylamide/3% glycerol gel was pre-run for 15 min prior to loading. In each lane 15 μ L of a 20 μ L reaction were loaded while the gel was running, to prevent the samples from being diluted. The gels were run at 4°C / 185 V. Gels were dried, exposed to a phosphorimager screen, and visualized using a Molecular Dynamics phosphorimager.

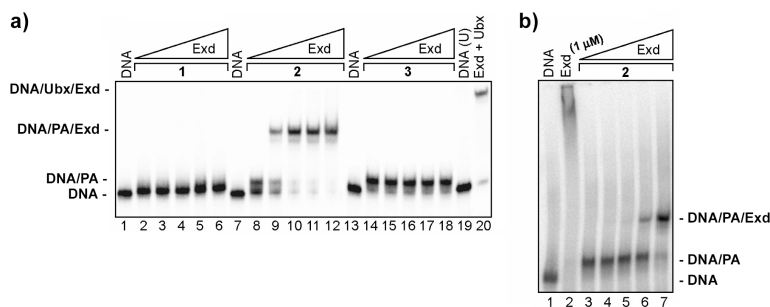


Figure S3. EMSA studies with polyamides **1-3**, Exd and Ubx. a) Each polyamide binds and decreases the mobility of free DNA (lanes 2-18). Compound **2** bearing the functional docking peptide is capable of recruiting Exd to DNA (lanes 9-12) whereas **1** & **3** are not. In lanes 2-6, 8-12, 14-18, Exd was added in following concentrations: 0, 3, 10, 30, 100 nM. Lanes 19 and 20 contained DNA bearing the Exd-Ubx binding site that was used in X-ray crystal structure determination (see methods above for sequence). In the reaction shown in lane 20, 275 nM Ubx and 30 nM Exd were incubated with DNA. b) Multiple Exd molecules bind DNA at 1 μ M concentration (lane 2), reactions in lanes 3-7 contain 50 nM PA **2** and increasing concentration of Exd (0, 0.3, 1, 3, 10 nM in lanes 3-7).

Gel-shift studies with the Ubx protein were performed under identical buffer conditions using the duplex oligonucleotide listed in Figure S2 (d). Ubx was added to the reaction mixture containing 32 P end-labeled duplex DNA and incubated at 4°C for 30 min. Subsequently Exd was added and the reaction was further incubated for 60 min at 4°C. The complexes were resolved under similar gel conditions as those described for DNA-polyamide-Exd complexes above. The K_d of Ubx for its cognate DNA was found to be 200 ± 25 nM. Under saturating concentrations of Ubx (325 nM) the affinity of Exd for the binary [Ubx-DNA] complex was 2-3 fold larger than the affinity of Exd for the conjugate **2**·DNA binary complex.⁶

References.

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