Appendix

Distinct modes of recruitment of the CCR4-NOT complex by *Drosophila* and vertebrate Nanos

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Contents

Appendix Supplementary Materials and Methods p. 2–6

Appendix References p. 7–8

Appendix Figures S1 and S2 p. 9–12

Appendix Tables S1 and S2 p. 13–15
Appendix Supplementary Materials and Methods

DNA constructs

Luciferase reporters and plasmids for the expression of GFP- or HA-tagged subunits of the CCR4-NOT and PAN2-PAN3 deadenylase complexes, decapping factors, DCP2 catalytic mutant (E361Q) and GW182 were previously described (Behm-Ansmant et al., 2006; Tritschler et al., 2008; Haas et al., 2010). An F-luc-hb reporter was generated by inserting the hunchback 3' UTR (CG9786) into the NheI and XhoI restriction sites of plasmid pAc5.1-F-Luc (Behm-Ansmant et al., 2006). Plasmids for the expression of GFP and λN-HA tagged Dm Nanos (Uniprot A0A0B4KGY5-1) were obtained by inserting the cDNA corresponding to the Nanos ORF into the EcoRI and XhoI sites of the pAC5.1-EGFP and pAC5.1-λNHA vectors (Behm-Ansmant et al., 2006). Nanos fragments were amplified by PCR using the full-length Nanos template and inserted into the same vectors. Deletion constructs were made by site-directed mutagenesis using appropriate primers. To generate the chimeric NIM-ZnF construct, we inserted a cDNA corresponding to the Hs Nanos2 NIM motif (codon-optimized for expression in Dm) followed by a Gly-Ser-Ser-Gly linker between the GST and Dm Nanos ZnF sequences of the pAC5.1-λNHA-GST-ZnF plasmid.

For the expression of recombinant proteins in E. coli, synthetic cDNAs (codon-optimized for expression in E. coli) corresponding to Dm Nanos fragments were inserted into the XhoI and BamHI restriction sites of the pnEA-pG plasmid (Diebold et al., 2011), generating protein fusions containing N-terminal GST tags cleavable by the HRV3C protease. The NED and NED-ΔNBR constructs contain a C-terminal GB1 tag (Chen and Patel, 2004) fused to the Nanos sequences by a Gly-Ser-Ser-Gly linker.

Plasmids for the expression of the Hs NOT1 SHD and the NOT2 and NOT3 C-terminal regions have been previously described (Boland et al., 2013; Bhandari et al., 2014). Human NOT2 and NOT3 were expressed from a bicistronic plasmid based on the pnEA vector.
(Diebold et al., 2011) and contained HRV3C-cleavable MBP and 6xHis tags, respectively. The DNA constructs used in this study are listed in Appendix Table S1.

**mRNA half-live**

For the measurement of mRNA half-lives, transfected cells were treated with actinomycin D (5µg/ml final concentration) 3 days after transfection, and harvested at the time points indicated. RNA samples were analyzed by Northern blot. mRNA reporter levels were normalized to the levels of rp49 mRNA and were plotted against time. The mRNA half-lives ($t_{1/2}$) ± standard deviations were calculated from the decay curves (not shown) obtained from three independent experiments and are indicated below the panels.

**Protein expression and purification**

All proteins for crystallization and *in vitro* pulldown assays were expressed in *E. coli* BL21 (DE3) Star cells (Invitrogen) in ZY medium at 20 °C overnight. *Dm* Nanos constructs were expressed with N-terminal GST tags. The NED constructs carried, in addition a C-terminal noncleavable GB1 tag. The cells were resuspended and lysed in binding buffer containing 50 mM HEPES (pH 7.5), 300 mM NaCl and 2 mM dithiothreitol (DTT) supplemented with protease inhibitors, lysozyme and DNaseI. The proteins were isolated from the crude lysate using Protino glutathione agarose 4B beads (Macherey Nagel) and eluted in binding buffer containing 25 mM glutathione. For GST pulldown assays, the proteins were further purified by anion exchange chromatography using a HiTrap Q column (GE Healthcare) followed by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in a buffer containing 10 mM HEPES (pH 7.5), 200 mM NaCl and 2 mM DTT. For crystallization, the GST tag was cleaved after elution from the glutathione beads by incubating overnight with
recombinant HRV3C protease. The protein was separated from the tag by gel filtration on a Superdex 75 26/60 column (GE Healthcare).

The assembled Hs NOT module was obtained by co-expression of MBP-tagged NOT1 SHD (residues 1833–2361), MBP-tagged NOT2 (residues 350–540) and His6-tagged NOT3 (residues 607–748). The cells were lysed in lysis buffer supplemented with DNaseI, lysozyme and protease inhibitors. The protein complex was purified over amylose resin and eluted with lysis buffer supplemented with 25 mM D-(+)-maltose and 20 mM imidazole. The NOT module was further purified via nickel affinity chromatography using a HiTrap IMAC column (GE Healthcare). The affinity tags were removed by overnight cleavage using HRV3C protease during dialysis in a buffer containing 50 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT. The cleaved MBP tags were removed by binding to amylose resin. The remaining contaminants were removed by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in a buffer containing 10 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT.

**Crystallization**

Initial screens were carried out using the sitting drop vapor diffusion method using 5 mg/ml of the Hs NOT module or a mixture containing the Hs NOT module (5 mg/ml) and a 1.5-fold molar excess of the Dm Nanos NBR peptide. Samples (200 nl) were preincubated for 1 hr in a buffer containing 10 mM Tris-HCl (pH 8.6), 200 mM NaCl, 10% glycerol and 2 mM DTT and were added to 200 nl of reservoir solution. Crystals appeared within three days in many different conditions containing polyethylene glycol (PEG). The best NOT module crystals were optimized to grow in 0.2 M sodium acetate, 0.1 M sodium citrate (pH 5.5) and 10% (w/v) PEG 4000. The best-diffracting crystals of the NOT module bound to the Nanos peptide were optimized to grow over a week in 100 mM MES (pH 6.0), 260 mM LiCl and 18.6% (w/v) PEG.
Crystals were cryoprotected using reservoir solution supplemented with 15% glycerol and flash-frozen in liquid nitrogen.

**Data collection and structure determination**

Diffraction data were recorded on a PILATUS 6M detector at the PXII beamline of the Swiss Light Source (SLS) at a temperature of 100 K. Data were processed using XDS and XSCALE (Kabsch, 2010). Initial phase information was obtained by molecular replacement with the structure of the *Hs* NOT module (PDB code 4C0D) as a search model using PHASER (McCoy *et al.*, 2007) from the CCP4 package (Winn *et al.*, 2011). The models were then improved by iterative cycles of refinement using PHENIX (Afonine *et al.*, 2012) and BUSTER (Bricogne *et al.*, 2011) and manual building in COOT (Emsley *et al.*, 2010). Finally, the *Dm* Nanos NBR was built into the density and improved by several additional refinement cycles. Reported coordinate errors (Table 1) are from BUSTER and correspond to the diffraction-component precision index (Blow, 2002).

**Anomalous difference Fourier map**

Anomalous data were also recorded at the PXII beamline, at a wavelength of 0.979 Å and to a resolution of 3.9 Å. Data were processed and scaled using XDS and XSCALE, keeping Friedel mates apart to extract the anomalous difference for the calculation of the map coefficients. Phases were obtained from molecular replacement (PHASER) using the refined structure of the complex and searching for two copies, followed by one cycle of rigid body refinement in PHENIX.
Sequence searches and alignments

Nanos and NOT1–3 protein sequences were retrieved from TREEFAM (http://www.treefam.org) and aligned using the MAFFT webserver (http://mafft.cbrc.jp; L-INS-i preset) from within JALVIEW (http://www.jalview.org). Positional conservation and similarity scores were calculated using the SCORECONS webserver (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl) with default settings. Alignments were illustrated manually.
Appendix References


Appendix Figure S1. Sequence alignment of the NOT1 SHD.

The secondary structural elements as determined from the *Hs* NOT1 structure are shown above the alignment. The residues conserved in all of the aligned sequences are shown on a dark magenta background, and the residues with >70% similarity are shown on a light magenta background. The residues interacting with the *Dm* NBR and the human Nanos1 NIM peptide are indicated by red and orange diamonds, respectively. The residues mutated in this study are indicated by asterisks colored in blue (mutations that disrupt NBR binding) or in green (crystallization mutations). Loop L19 as observed in the previous structure of the NOT module (PDB entry 4C0D) folds as an α-helix (α22’) in the present structures. The species abbreviations are as follows: *Hs* (*Homo sapiens*), *Dm* (*Drosophila melanogaster*), *Ag* (*Anopheles gambiae*), *Bm* (*Bombyx mori*), *Dr* (*Danio rerio*), and *Ce* (*Caenorhabditis elegans*).
Appendix Figure S2. Sequence alignment of the NOT2 and NOT3 C-terminal fragments.

A NOT2

The secondary structural elements as determined from the Hs NOT module structure are shown above the alignment. The residues conserved in all of the aligned sequences are shown with a dark green (NOT2) or cyan (NOT3) background, and the residues with >70% similarity are highlighted with a light green or cyan background. The NOT3 residues
interacting with the *Dm* NBR are indicated by red diamonds. The residues mutated in this study are indicated by blue asterisks. The species abbreviations are as described in Appendix Fig S1.
## Appendix Table S1. Constructs used in this study.

<table>
<thead>
<tr>
<th><strong>Dm</strong> Nanos, isoform B (Uniprot A0A0B4KGY5-1)</th>
<th>Comment</th>
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</table>
| Full length | λN-HA-Nanos  
GFP-Nanos |
| ΔZnF | λN-HA-Nanos 1–296  
GFP-Nanos 1–296 |
| ZnF | λN-HA-GST-Nanos 297–381  
GFP-Nanos 297–381 |
| NED | GST-Nanos 50–236-GB1  
λN-HA-GST-Nanos 50–236  
GFP-Nanos 50–236 |
| Nanos-ΔNED | λN-HA-Nanos Δ50–236  
GFP-Nanos Δ50–236 |
| NED ΔNBR | GST-Nanos 50–236-Δ116–163-GB1 |
| NBR | GST-Nanos 116–163  
λN-HA-GST-Nanos 116–163 |
| NBR 3xMut | GST-Nanos 116–163  
E151A,F152A,N155A |
| NBR F152E | GST-Nanos 116–163 F152E  
E151A,F152A,N155A |
| NBR 2xMut | GST-Nanos 116–163 L127D,F130D |
| NBR I123M | GST-Nanos 116–163,1123M  
SeMet labeling |
| 50–115 | λN-HA-GST-Nanos 50–115  
164–236 | λN-HA-GST-Nanos 164–236 |

| **Hs** Nanos2 (Uniprot P60321) - **Dm** Nanos chimera |
|---|---|
| NIM-ZnF | λN-HA-GST-Hs Nanos2 NIM-Dm  
Nanos 297–381 |
| NIM’ZnF | λN-HA-GST-HsNanos2 NIM  
(F6A,W9E)-DmNanos 297–381 |

| **Hs** Nanos3 (Uniprot P60323) |
|---|---|
| NIM | MBP-Hs Nanos3 4–20-Strep |

| **Hs NOT1** (CNOT1, Uniprot A5YKK6) |
|---|---|
| MBP-NOT1-SHD | MBP-Hs NOT1 1833–2361 |
| MBP-NOT1-SHD EEE | MBP-Hs NOT1 1833–2361  
H2344E,C2345E,A2346E |
| MBP-NOT1-SHD V1880E | MBP-Hs NOT1 1833–2361 V1880E |
| MBP-NOT1-SHD H1949D | MBP-Hs NOT1 1833–2361 H1949D |

<p>| <strong>Hs NOT2</strong> (CNOT2, Uniprot Q9NZN8) |
|---|---|
| NOT2-C | MBP-Hs NOT2 350–540 |</p>
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<td>6xHis-<strong>Hs NOT3</strong> 607–748</td>
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<tr>
<td>NOT3-C Y702A</td>
<td>6xHis-<strong>Hs NOT3</strong> 607–748 Y702A</td>
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<tr>
<td>NOT1-C V2002E</td>
<td>λN-HA-NOT1 1710–2505 V2002E</td>
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<tr>
<td>NOT1-C H2067D</td>
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<td>NOT3-L</td>
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<td>NOT3-C</td>
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<td>NOT2-C</td>
<td>λN-HA-NOT2 402–585</td>
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<td>F-Luc-<em>hunchback</em> 3’UTR</td>
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<td>F-Luc-<em>hunchback</em>-3’UTR Δ(2495–2499)nt</td>
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<td>Δ(2546–2550)nt</td>
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<tr>
<td>ΔBoxB</td>
<td>F-Luc-<em>hunchback</em>-3’UTR Δ(2505–2510)nt</td>
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<td>Δ(2556–2561)nt</td>
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### Appendix Table S2. Antibodies used in this study.

<table>
<thead>
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<th>Antibody</th>
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<th>Monoclonal/ Polyclonal</th>
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<td>Anti-HA-HRP (for western blots)</td>
<td>Roche</td>
<td>12 013 819 001</td>
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<td>Monoclonal</td>
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<tr>
<td>Anti-HA (for immunoprecipitations)</td>
<td>Covance</td>
<td>MMS-101P</td>
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<td>Mouse monoclonal</td>
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<tr>
<td>Anti-GFP (for western blots)</td>
<td>Roche</td>
<td>11 814 460 001</td>
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<td>Mouse monoclonal</td>
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<tr>
<td>Anti-GFP (for immunoprecipitations)</td>
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<td>Tritschler et al, 2008</td>
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<tr>
<td>Anti-mouse IgG-HRP</td>
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<td>NA931V</td>
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<td>Anti-rabbit IgG-HRP</td>
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<td>Donkey polyclonal</td>
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