Blot hybridizations
For the Southern blot, 4 µg of genomic DNA from each of nine eukaryotes was digested with EcoRI, separated on a 0.7% agarose gel, transferred onto nylon membrane and hybridized overnight at 65 °C in 6 x SSC/0.5% SDS using a 32P-radiolabelled synctin coding region probe (200 base pairs). The filter was washed in 2 x SSC/0.5% SDS and in 0.2 x SSC/0.1% SDS, both for 2 h at 65 °C, before X-ray film exposure. For northern blots hybridizations and washes were as described above, but at 72 °C.

In situ hybridization of placental tissue
Sections were prepared and processed as described, and were probed with digoxigenin-labeled synctin antisense and sense RNA. Sections were stained using the alkaline phosphate substrates NBT and BCIP using conditions recommended by the manufacturer.

Fused COS-1 cell staining
COS-1 cells were stained 48 h after transfection. Cells were washed with PBS, air dried, fixed with Diff-Quick fixative (10 min), treated with Diff-Quick solution I (10 min), washed again with PBS, stained with Diff-Quick solution II (2 min), washed again with PBS and visualized under a light microscope.

IL-12 PHA blast assay
PHA (phytohaemagglutinin) blasts were prepared by stimulating human peripheral blood cells with PHA for four days, followed by three days with PHA and IL-12, and were then used to measure bioactive IL-12 in a proliferation assay.

Liposome cell fusion
We mixed 2 mg heart lipid extract with 5 µg GFP expression plasmid in 1 ml PBS. The mixture was homogenized (10 min) with a hand-held homogenizer and centrifuged (30 min) at 8 000g. The pellet, resuspended in PBS, was added to COS cells expressing synctin. GFP expression was detected after 48 h by fluorescence microscopy.

Received 29 April; accepted 20 December 1999.


Acknowledgements
We thank the Genetics Institute signal sequence trap team for the initial cloning of synctin; J. Wooters for help in liposome preparations; B. Gimlich, M. Ouattara and the Genetics Institute Developmental Biology group for microscopy assistance; the Genetics Institute DNA synthesis group for oligonucleotides; and M. Davies, R. Piujnornberg and K. Turner for critical review of this manuscript.

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During animal development, cells have to respond appropriately to localized secreted signals. Proper responses to Hedgehog, transforming growth-factor-β, epidermal growth factor and fibroblast growth factor/Ras signals require cognate inducible antagonists such as Patched, Dad, Arpgo and Sproul. Wnt signals are crucial in development and neoplasia. Here we show that naked cuticle (nkd), a Drosophila segment polarity gene, encodes an inducible antagonist of Wnt signalling. In fly embryos and imaginal discs nkd transcription is induced by Wg. In embryos, decreased nkd function has an effect similar to excess Wg; at later stages such a decrease appears to have no effect. Conversely, overproduction of Nkd in Drosophila and misexpression of Nkd in the vertebrate Xenopus laevis result in phenotypes resembling those of loss of Wg/Wnt function. nkd encodes a protein with a single EF hand (a calcium-binding motif) that is most similar to the recoverin family of myristoyl switch proteins. Nkd may therefore link ion fluxes to the regulation of the potency, duration or distribution of Wnt signals. Signal-inducible feedback antagonists such as nkd may limit the effects of Wnt proteins in development and disease.

Wg is critical for patterning events during the three stages of embryonic segmentation in Drosophila. First, 3–3.5 h after egg laying (AEL), adjacent stripes of cells produce Wg and SonC1 (Hh). Engrailed (En) and Hh are produced in the same cells. Second, between 3.5 and 6 h AEL, Wg maintains hh/en transcription and Hh maintains Wg transcription, producing a transient segmentation landmark, the parasegmental groove. Finally, from 6 h AEL
on, definitive segmentation results in ventral epidermal cells synthesizing a segmentally repeated trapezoidal array of six unique types of cell protrusion called denticles, interspersed with naked (dentine-free) cuticle. During this stage, Wg specifies naked cuticle fates. \( nkd \) is an embryonic lethal recessive zygotic mutation that produces multiple segmentation defects, the most prominent of which is the replacement of denticles by excess naked cuticle (Fig. 1a, d). This phenotype is also seen in embryos exposed to excess Wg, as well as in embryos lacking both maternal and zygotic contributions from any of three genes that antagonize Wg: zeste-white3/glycogen synthase kinase 3 \( \beta \) (zw3/gsk3\( \beta \)) (ref. 8), D-axin (ref. 9) and D-Apc2 (ref. 10). In \( nkd \) embryos, hh and en transcripts initiate normally but accumulate in broad stripes including cells further from the source of Wg, as if those cells are hypersensitive to Wg (Fig. 1e). Next, a stripe of new Wg transcription appears just posterior to the expanded Hh/En stripe (Fig. 1f). This extra Wg stripe requires both Wg and hh activity and is required for the excess naked cuticle seen in \( nkd \) mutants. The death of cells producing Hh/En contributes to the marked shortening of \( nkd \) mutant cuticles (Fig. 1d).

To clone \( nkd \), we identified a fly stock with a \( lacZ \) P-element insertion in the \( nkd \) gene. We used DNA adjacent to the P insert to

**Figure 1** nkd embryonic phenotype and rescue by nkd cDNA. Wild-type ventral cuticle (a) and stage 11 embryonic En (b) and Wg (c) stains. Homozygous strong nkd mutants (nkd\( ^{7H16} \) or nkd\( ^{7E89} \)) have excess naked cuticle (d), widened En staining (e; black arrowheads) and normally spaced endogenous wg stripes (f; black arrows) interspersed with ectopic wg stripes (red arrows), most commonly in alternate parasegments. Ubiquitous Nkd expression in homozygous nkd\( ^{7H16} \) or nkd\( ^{7E89} \) embryos rescues the naked cuticle phenotype (g), narrows En stripes to 2–3 cells wide (h) and restores the wild-type Wg expression pattern (i). Homozygous mutant nkd embryos are identified by the absence of hunchback–lacZ expression (red arrowheads) on the TM3 balancer chromosome. The most potent rescue was observed when 2–4 h P[\( Hs-nkd \); nkd\( ^{7H16} \)/TM3 embryos were heat shocked for 15 min at 37 \( ^\circ \)C, which resulted in 0% 'strong', 12% 'moderate', 12% 'weak' and 76% wild-type cuticles (n = 101) (see Methods for cuticle scoring). Scale bar, 60 \( \mu \)m.

**Figure 2** The nkd gene. a, Genomic map of nkd relative to centromere (cen) and telomere (tel) of chromosome 3L, band 75F. P-element transposon \( l(3)4869 \) is upstream of exon 1. nkd has five exons with a \( \approx 25 \)-kb first intron. The putative initiator methionine (M) is near the 3' end of the first exon, whereas the stop codon (X) is in the middle of exon 5. A nonsense mutation at codon 60 (arrow above exon 2) was found in nkd\( ^{7H16} \).

b, Developmental embryonic northern blot. Hours AEL shown above each lane.

c, Immunoprecipitation of Nkd from 3–8-h embryonic extracts by anti-Nkd antibody. pre, preimmune antisera; Anti-Nkd, immune affinity purified anti-Nkd antisera; S, supernatant; P, pellet. A specific band (arrow) migrates above the 121K marker.

d, Alignment of EF-hand similarity between Nkd and third EF hand in recoverin family of proteins. Amino-acid identities between Drosophila Nkd (Dm. Nkd), Drosophila Neurocalcin (Dm. Ncalc; Genbank accession no. 1171668) and bovine Recoverin (Bov. Rec; Genbank accession no. 494545) are designated by vertical bars. Consensus EF-hand residues (EF cons) are shown in key symbols used by Stryer (above) or Kretsinger (below)\( ^{30} \). h, hydrophobic residue; E, acidic residue, usually glutamic acid; O, oxygen-donating residue that binds Ca\( ^{2+} \); G, glycine; asterisk, variable amino acid; J, hydrophobic residue; X, Y, Z, coordinates of Ca\( ^{2+} \) binding in three-dimensional space.
probe complementary DNA and genomic libraries, resulting in a genomic DNA walk of about 80 kilobases (kb) (Fig. 2a). A single 5-kb messenger RNA transcript derived from 40 kb of genomic DNA is expressed zygotically in a striped pattern (Figs 2a, b and 3). Consistent with the lack of a maternal requirement for nkd (S. DiNardo, personal communication), the transcript is absent from maternally derived embryonic RNA from 0–2 h AEL (Fig. 2b). The longest cDNA, 4,954 base pairs (bp), has an open reading frame (ORF) of 2,784 bp, encoding a 928-amino-acid, relatively basic protein. Single-stranded conformation polymorphism (SSCP) analysis and direct genomic sequencing reveals a nonsense mutation Q60stop in nkdH16 (SSCP), predicting a truncated protein of 59 amino acids (Fig. 2a). The identity of the nkd cDNA was further confirmed by its ability, when activated with a heat-shock promoter, to rescue the naked cuticle phenotype and the En and Wg expression abnormalities in nkd mutants (Fig. 1o–i).

Nkd has significant similarity to the high-affinity Ca\(^{2+}\)-binding EF hand of the recoverin family of myristoyl switch proteins (Fig. 2d; 39% amino-acid identity, 63% similarity with Drosophila neurecalcin, \(P = 5 \times 10^{-6}\)). EF hands are conserved Ca\(^{2+}\)-binding motifs that usually occur in pairs, although they have been observed singly. Mouse and human expressed sequence tag clones encoding EF-hand sequences similar to fly Nkd homologues, a possibility we are currently testing. No obvious homologue has been identified in Caenorhabditis elegans. Affinity-purified anti-Nkd antisera made against either of two parts of the protein detect a segmentally repeated cytoplasmic distribution very similar to the embryonic RNA pattern (Fig. 3a, b). No staining is detected in nkd\(^{MIN}\) mutant embryos, and high-level ubiquitous expression is seen in heat-shocked P[Hs-nkd] embryos (Fig. 3b). Nkd antibody immunoprecipitates from embryonic protein extracts a protein that runs at a slightly higher relative molecular mass (\(M_r\)) than its predicted \(M_r\) of 102,000 (102K) (Fig. 2c).

Nkd transcription is initiated in embryos during the late cellular blastoderm stage in broad anterior and posterior domains, in a manner reminiscent of gap genes (Fig. 3a, stage 6). During early germ-band extension (stage 8–9), nkd transcription is nearly ubiquitous, with higher RNA levels in the 2–3 cell rows posterior to the Hh/En stripe that require nkd to limit Hh/En production (Fig. 3a, stage 9). At this stage, Wg protein is evenly distributed on both sides of the stripe of cells that express wg RNA. During full germ-band extension, nkd expression is most abundant anterior to, and lower just posterior to, the Hh/En stripe (Fig. 3a, stage 10–11). nkd RNA is lower still in the Hh/En-producing cells. Hh signalling in the Hh/En cells excludes Wg protein during this time, resulting in asymmetric Wg distribution with an anterior bias. nkd mutants do not develop this anterior bias of Wg\(^{a\,b}\), indicating that nkd may be required for hh to exclude Wg from Hh/En cells. Nonetheless, after embryonic stage 10, Wg protein and nkd RNA are found together in many tissues. nkd RNA and Wg RNA are expressed in overlapping patterns in imaginal discs and other larval tissues, with nkd domains being slightly broader than those of wg (Fig. 3d).

We tested whether nkd is regulated by Wg activity using gain- and loss-of-function experiments. In wg mutant embryos, nkd tran-

Figure 3 nkd expression and dependence on wg. a, nkd expression during embryonic stages 6–7 (left), 9 (middle) and 11 (right); anterior is left in all panels. Stage 6 nkd RNA (top) and Nkd protein (Anti-Nkd; middle) patterns are similar. Bottom, embryo labelled for En protein (brown) and nkd RNA (purple). Stages 9 and 11: nkd RNA with and without En protein patterns at low (top row) and higher power (bottom two rows). nkd accumulates posterior to the Hh/En stripe during stage 9 (red bracket). Later (stage 11), nkd RNA is highest in the 2–3-cell rows anterior to the Hh/En stripe (white bracket), with lower levels just posterior to the En stripe (red bracket). Parasegmental grooves are marked with red arrowheads in a and b. Anti-Nkd antiserum does not stain nkd\(^{MIN}\) embryos (left) and stains all cells after P[Hs-nkd] embryos are briefly heat pulsed at 37 °C (middle left). Wild-type stage 7 (middle right) and 11 (right) embryos stained with anti-Nkd antiserum reveal cytoplasmic and plasma membrane-associated (inset, arrowheads) epidermal staining similar to the RNA pattern. Asterisk, nucleus; brackets as in a, c, nkd in situ hybridization to stage 11 wild-type (+), wg and nkd mutant embryos. b, Regulation of nkd expression by wg in third instar wing pouch (left) and leg (right) imaginal discs. Top: wg RNA defines the presumptive wing margin and ventral-anterior sector of the leg disc (blue arrowheads). Middle: nkd expression (red arrowheads) is broader than that of wg. Bottom: Ecotopic nkd (red arrows) accumulates perpendicularly to wing margin or in the dorsal leg disc when dpp–Gal4 transgene drives UAS–wg. Scale bar, 85 μm in a left and top, b left two panels and c; 35 μm in a right and bottom, b right two panels and d left; 7 μm in b middle right inset; and 70 μm in d right.
scription initiates normally but is markedly reduced by stage 11 (Fig. 3c). nkd transcript accumulates to higher levels and more broadly across the segment in nkd mutant embryos (Fig. 3c), presumably owing to the lack of negative feedback that Nkd protein normally provides to its own Wg-dependent expression. nkd expression is enhanced when Wg is ubiquitously expressed (not shown). Misexpression of either Wg or an activated form of the Wg signal transducer Armadillo (UAS-ArmG141) in wing, leg, haltere and antennal imaginal discs results in similar patterns of ectopic nkd transcription (Fig. 3d). ArmG141-induced nkd transcript obeys sharp boundaries consistent with a cell-autonomous induction of nkd by Wg (not shown).

If loss of nkd mimics the effect of excess Wg, then excess Nkd should mimic loss of Wg. When P(Hs-nkd) is used to overexpress nkd in otherwise wild-type embryos, rare cuticles with weak wg-like denticle belt fusion phenotypes are observed (not shown), similar to those seen when zw3 is overexpressed16. Nkd is more potent in a sensitized wg+/background. In wg+/+ embryos, induction of P(Hs-nkd) before 4 h AEL results in decreased en and wg expression (not shown). wg+/+ embryos are patterned normally, but practically all wg+/+ embryos exposed to high levels of Nkd secrete cuticles with denticle belt fusions and an excess of the predominant denticle type made by wg/wg embryos (Fig. 4a, b).

Misexpressing nkd during larval development using UAS/Gal4 transgenic results in adult phenotypes that are indistinguishable from many wg loss-of-function phenotypes. The phenotypes that we observed include (1) wing-to-notum transformations18,19; (2) leg truncations (Fig. 4e) and duplications20 (Fig. 4f); (3) loss, lateral displacement and disorientation of sternite bristles21; (4) haltere loss21; (5) ventral eyereduction21; (6) loss of wing margin22; (7) extra wing anterior crossoves (C. Conley and S. Blair, personal communication); and (8) loss of antenna21. The gene dosage of the Wg pathway influences the effect of ectopic Nkd: loss of one wild-type copy of porcupine (porc), wg, dishevelled (dsh) or arm, enhances, and

![Figure 4](image_url) Consequences of altered nkd activity. a, Ubiquitous Nkd expression using P(Hs-nkd) in wg/+ embryos before 4 h AEL results in shortened embryos (left, dark field) with denticle belt fusions and excess type 5 denticles (right, and magnified inset). b, wg/wg mutant embryos display a uniform lawn of type 5 denticles and are unaffected by excess nkd. c, X-gal-stained dpp–lacZ leg disc. dpp is expressed along the A–P axis at high levels dorsally (red arrow) and lower levels ventrally (black arrow). d, E105 - Gal4; UAS–nkd imaginal discs have equally high levels of dpp–lacZ expression dorsally and ventrally (red arrows). e, E105 - Gal4; UAS–nkd legs are variably truncated. f, E119–Ga4;UAS–nkd legs are variably duplicated. g, h, Phenotypically normal nkdTE89 mutant wing margin (g) and leg clone (h) marked with the bristle marker yellow. i, Leg disc fate map with sectors of unique bristle identities labelled as follows: green, anterior + anterior dorsal; blue, dorsal + posterior dorsal; lavender, posterior + posterior ventral; yellow, ventral + anterior ventral. Bristles in tibial and tarsal leg segments were scored. Perimeter of approximate domains of Wg (red) and nkd (blue) expression are designated with lined. j, Per cent of nkdTE89 clones (n = 39; coloured bars) and control clones (n = 28; black bars) as a function of leg disc quadrant as shown in i. Within each sector, nkd and control clones occur with comparable frequency. k–m, Negative (k,l) and positive (m) confocal microscopic images of horizontally oriented wing disc margin harbouing multiple nkdE323 clones (marked by lack of P–myc stain in l; green in merged image of m) stained with anti-Dll [k, red in m]. Representative clone spanning region of greatest drop-off in Dll stain is noted by a yellow line around its perimeter, highlighted by arrows in l. Twin spots are marked by more intense P–myc stain throughout the disc (l,m). Scale bar, 110 μm in a and b, left; 40 μm in c and d; 120 μm in e–h; 35 μm in k–m.

![Figure 5](image_url) Misexpression of fly Nkd in Xenopus embryos and animal caps. Embryos with shortened A–P axes produced by injection of 2.0 ng Drosophila nkd RNA (a) or the dominant inhibitory Xfz8 (b) into dorsal blastomere at 4-cell stage. c, Injection of water has no effect. Animal caps explanted from embryos injected with activin RNA (d) elongate (red arrow) when cultured. Simultaneous expression of activin and nkd RNA (e) blocks elongation. nkd RNA injected without activin has no effect on animal cap elongation (not shown). f, Embryos receiving ventral blastomere injection of 0.5 pg Xwnt8 RNA produce partial duplicated axis (white arrow). g, Embryos injected with 0.5 pg Xwnt8 and 7 pg fly nkd do not develop ectopic axes. h, Ventral injection of 2 ng nkd RNA results in head duplication. Note the duplicated eye and cement gland (cg with arrow) and duplicated abdominal pigmentation (dashed line). The image in h has been previously published15. Scale bar, 750 μm in a–c, f–h; 375 μm in d and e.
loss of zw3 and nkd suppresses, the UAS–nkd overexpression phenotypes (data not shown).

During leg development, Wg and Decapentaplegic (Dpp; a signalling protein related to vertebrate bone morphogenetic proteins) act as mutually antagonistic determinants of dorsal and ventral identity\(^1\). Dpp is expressed at high levels at the anterior–posterior (A–P) boundary dorsally, and at lower levels ventrally (Fig. 4c). Wg/Dpp juxtaposition results in leg disc eversion and outgrowth during pupal morphogenesis. Excess Nkd expressed throughout the disc results in high levels of laZ expression along the entire A–P border of the discs (Fig. 4d), and scant wg–laZ expression (not shown), similar to that seen when wg activity is reduced\(^2\). These discs give rise to variably truncated legs (Fig. 4e), indicating that excess Nkd can antagonize the normal effects of Wg. More restricted ventral Nkd misexpression results in duplicated legs (Fig. 4f), which may arise by an abnormal juxtaposition of cells still expressing Wg and cells in which excess Nkd results in decreased Wg, and hence derailed dpp. Thus two Wg/Dpp boundaries are created and appendages are duplicated.

We induced the loss-of-function clones in imaginal discs and adult structures using two strong nkd alleles, nkd\(^{9G33}\) and nkd\(^{7E89}\), and one moderately severe allele, nkd\(^{7H16}\), all of which are embryonic lethal. nkd alleles were originally generated in the genetic background of a weak allele of the pair-rule gene hairy (h\(^+\)). h\(^+\) clones give rise to ectopic wing-vein bristles and thoracic microchaetes. Furthermore, nkd and h genetically interact: nkd, h\(^+/h^{null}\) is lethal, whereas h\(^+/h^{null}\) is viable (A. Martinez-Arias, personal communication). Therefore we also generated clones of the strong allele nkd\(^{7E89}\) from which h\(^+\) had been removed. In many tissues where Wg signals control pattern, including the wing (Fig. 4g), leg (Fig. 4h), thorax, abdomen, haltere and eye, we observed phenotypically normal nkd clones. h\(^+\), nkd\(^{9G16}\) but not h\(^+\), nkd\(^{7E89}\) or h\(^+\), nkd\(^{7G33}\) clones give rise to a rough eye phenotype and loss of wing margin bristle phenotype (not shown), which may be due to h–nkd interactions.

To test whether nkd clones arise at biased locations, we scored clones in adult legs marked with the bristle marker yellow (Fig. 4i,j). nkd and control clones appeared with similar frequency in each leg quadrant (Fig. 4j). We assayed the expression of Wg target genes in nkd clones to look for subtle changes in gene expression that might be compatible with normal tissue patterning. Distalless (Dll) is distributed in a broad gradient centred on the margin of the wing disc (Fig. 4k). Induction of nkd clones results in no apparent alteration in the Dll expression gradient within, or adjacent to, multiple clones (Fig. 4k–m). No changes in cytoplasmic Arm accumulation, an indicator of Wg activity, are noted within or adjacent to nkd clones (data not shown).

We investigated whether fly nkd can alter Wnt signalling in a vertebrate by injecting mRNA into Xenopus embryos. Dorsal blastomere injection into 4-cell embryos of RNAs encoding the Wnt antagonist FrzB\(^24\), as well as dominant inhibitory forms of Xfz8\(^{25,26}\) (Fig. 5b), Dishevelled (Xdd)\(^{27}\) and Wnt-8\(^{25}\), blocks cell movements that drive the elongation of the gastrula and neurula\(^{25,27}\). These movements drive the elongation of ectodermal explants (animal caps) induced to form mesoderm by activin (Fig. 5d). Wnt antagonists (such as NXfz8 or Xdd) block elongation of explants without inhibiting mesoderm induction\(^{25,27}\). Injection of 2.0 ng nkd RNA into animal caps mimics this inhibitory effect, blocking elongation in response to activin (2.5 pg mRNA) in 90% of explants (n = 47) (Fig. 5e).

Ectopic ventral expression of Wnts before the onset of zygotic transcription results in duplication of the dorsal axis, which can be blocked by Wnt antagonists such as NXfz8 (ref. 25), Xdd\(^{27}\) or FrzB\(^{24}\). nkd also blocks Wnt-mediated axis duplication. Ventral blastomere injection of 0.5 pg XWnt8 RNA resulted in ectopic dorsal axes in 56% of embryos (n = 88) (Fig. 5f), about half of which formed complete anterior structures. Co-injection of 3.5 pg nkd RNA reduced the frequency of ectopic axes to 42% (n = 72), with only 10% of embryos forming complete anterior structures. Co-injection of 35 pg nkd RNA gave only 19% (n = 80) partial secondary axes, with no complete axes. At these doses, nkd RNA alone has no discernible effect on development when injected into either dorsal or ventral blastomeres. Higher doses (350 pg–2 ng) of nkd RNA injected alone into ventral blastomeres give rise to ectopic heads (Fig. 5h), complete with eyes and cement glands. This striking phenotype has been attributed to antagonism of Wnt activity\(^{28,29}\).

Our data show that nkd antagonizes Wg/Wnt signalling. Does nkd affect Wnt synthesis or transport, or determine how cells respond to Wnt? Because Wg and other Wnts are autoregulatory in many contexts, Nkd could affect the quantity or distribution of Wg either directly by controlling Wg synthesis or transport, or indirectly by reducing the positive feedback of Wg activity on its own synthesis. Unfortunately, the lack of a nkd clone phenotype in the fly precludes definitive determination of cell-autonomous function. However, the first known gene expression defect in nkd mutants is in cells distant to Wg producers, indicating that nkd may act first in Wg-receiving cells. In addition, nkd RNA injected into Xenopus embryos does not alter the accumulation of epitope-tagged XWnt8 (not shown), indicating that nkd may block the response to XWnt8.

Inducible antagonists limit the effective duration, range of action or activity of a signal, and can act cell autonomously or non-autonomously\(^{31}\). Near-saturating genetic screen have revealed that nkd and patched (ptc; an inducible antagonist for Hh signalling) are probably the only Drosophila Wg or Hh pathway genes, other than Wg or Hh themselves, whose expression and genetic requirement are exclusively zygotic. All other known components of both signalling pathways are maternally provided. Evolutionary selective pressure apparently resists duplications of zygotically active inducible antagonist genes. Antagonist gene dosage must be carefully regulated in flies and vertebrates to balance the effects of the signals. In Drosophila, nkd and ptc mutations have haplo-insufficient effects on cuticle pattern in combination with each other and with other segment-polarity mutants\(^{21}\). Altered regulation of both Wnt and Hh signalling in mice and humans is implicated in precancerous and cancerous cell growth. Just as vertebrate ptc1 regulates cell fates and is a key tumour-suppressor gene, vertebrate Nkd-like proteins may be essential for restraining Wnt activity during development and possibly cancer progression.

Methods

Cloning of nkd

nkd\(^{9G33}\) and nkd\(^{7E89}\) fail to complement the lethality of enhancer trap line I(3)I-4869. Homozygous I(3)I-4869 embryos have features of weak nkd alleles (see below). Precise excision of the I(3)I-4869 P element by transposase eliminated lethality and the weak nkd mutant phenotype. A 4.9 kb Xba–Xba genomic DNA fragment immediately 3’ to the P-element insertion site in I(3)I-4869 was cloned by plasmid rescue and used to initiate the isolation of 80 kb of genomic DNA.

SSCP analysis

We performed SSCP analysis using MDE high-resolution gel from AT Biochem. 150 ng of genomic DNA was used as the template. Primers 100–700 bp apart were used for SSCP–PCR. The sequences of the two primers that detect a nonsense mutation at codon 60 in nkd\(^{9G33}\) are 5’-GCTGCTGCCAGGAAG-3’ (CTP18) and 5’-TGGATAAGCTGTCTTAC-3’ (CTP17).

Fly stocks and P-element-mediated transformation

w\(^{1118}\) was used at non-permissive temperatures, mimicking a null wg allele, in Figs 3c and 4a,b. P-element-mediated transformation was performed on yw flies, using P=Δ2-3 as a source of transposase.
Heat-shock mediated Nkd overexpression and nkd rescue

We made P[H-s-nkd] by cloning the Kpn–Kpn fragment from nkd cDNA clone C5 into Kpn-cut pBACAL, which harbour the hsp70 promoter and 3' untranslated regions. The P[H-s-nkd] construct that was used for rescue lacks 5' and 3' untranslated regions and has a carboxy-terminal 9-amino-acid human c-myc tag. Two independent transformant lines gave quantitatively similar results in Nkd overexpression experiments in both nkd mutant and w+ backgrounds. Most experiments used nkd100, nkd102 was rescued to a similar degree and nkd371, an independent allele of P[H-s-nkd], known nkd100 lethal, and their cuticle phenotype severity can be scored as follows. Strong' nkd mutants (such as nkd106 and nkd108) secrete cuticles with a fully exteriorized head skeleton, widely split fzkotor (posterior spiracles) and residual denticles only in A3 and/or A5 (or none at all), and are typically less than 75% of wild-type length. 'Weak' cuticles (such as nkd9H52 and nkd9G3) secrete cuticles, but possess largely normal fzkotor and head skeleton, and are almost wild-type length. 'Moderate' cuticles (such as nkd7E89 and nkd9H52) have a phenotype between weak and strong.

Nkd antibody production

Recombinant Nkd protein was made as a Trp–Nkd fusion. The fusion protein was purified from BL21 pLY5 Escherichia coli lysates as inclusion bodies, cut from an SDS–polyacrylamide gel and injected with adjuvant into rabbits (Iomam Labs). We made GST–Nkd fusion proteins to affinity purify anti-Nkd antisera by cloning similar fragments of a GST–nkd C terminus expressed in BL21 pLysS (D. Turner and R. Rupp) and mRNA was prepared by in vitro transcription using a cloned nkd mRNA with or without Xwnt8 mRNA into the animal pole of dorsal or ventral blastomeres at the 4-cell stage (10 nl per cell) and cultured embryos until the tadpole stage for scoring. For mesoderm induction assays, 10 nl nkd mRNA (0.2 ng nl–1) and/or activin (0.25 pg nl–1) mRNA was injected into the animal pole of fertilized eggs. At the blastula stage, animal caps were explanted, cultured until siblings reached the neurula stage and then scored for elongation.

Received 5 October; accepted 6 December 1999.


Acknowledgements

We thank L. Mathies for identifying the P element in the nkd gene. B. Cadigan, K. Carr, A. Carpenter, C. Donovan, N. Ito, C. Kirkpatrick, A. Martinez Arias, R. Nusse, C. Nüsslein-Volhard, G. Panganaban, N. Patel, M. Pfeifer, N. Perrimon, G. Rubin and A. Spradling for mutants, reagents and advice. J. Axelrod, L. Luo, R. Nusse and R. Rousset for comments on the manuscript; S. Blair, S. Conley, S. DiNardo, A. Martinez Arias, S. Sokol and M. Deardord for providing information before publication; M. Fish for
Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase

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Yeast Sir2 is a heterochromatin component that silences transcription at silent mating loci, telomeres and the ribosomal DNA, and that also suppresses recombination in the rDNA and extends replicative life span. Mutational studies indicate that lysine 16 in the amino-terminal tail of histone H4 and lysines 9, 14 and 18 in H3 are critically important in silencing, whereas lysines 5, 8 and 12 of H4 have more redundant functions. Lysines 9 and 14 of histone H3 and lysines 5, 8 and 16 of H4 are acetylated in active chromatin and hypoacetylated in silenced chromatin, and overexpression of Sir2 promotes global deacetylation of histones, indicating that Sir2 may be a histone deacetylase. Deacetylation of lysine 16 of H4 is necessary for binding the silencing protein, Sir3. Here we show that yeast and mouse Sir2 proteins are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases, which deacetyl ate lysines 9 and 14 of H3 and specifically lysine 16 of H4. Our analysis of two Sir2 mutations supports the idea that this deacetylase activity accounts for silencing, recombination suppression and extension of life span in vivo. These findings provide a molecular framework of NAD-dependent histone deacetylation that connects metabolism, genomic silencing and ageing in yeast and, perhaps, in higher eukaryotes.

Sir2 is a limiting component that promotes longevity in yeast mother cells. Cells lacking Sir2 have a reduced replicative life span and cells with an extra copy of Sir2 display a much longer life span than wild type. This extension probably results from a hypersilencing in the rDNA which reduces recombination and the production of extrachromosomal rDNA circles, a known cause of senescence in ageing mother cells. Sir2 homologues have been identified in many organisms ranging from bacteria to humans. The Salmonella homologue, cobB, has been implicated in a pyrimidine transfer reaction and both CobB and eukaryotic Sir2 proteins possess ADP-ribosyltransferase activity.

Because Sir2 proteins use NAD as a substrate in a ADP-ribosylation reaction, we examined whether NAD could be a co-factor necessary for deacetylase activity. We used purified recombinant Sir2 in a reaction with NAD and a peptide of the histone H3 N-terminal tail (residues 1–20) di-acetylated at lysines 9 and 14. We incubated 5 µg of recombinant yeast Sir2 (79 pmoles) (Fig. 1a), 10 µg of the H3 peptide (4.2 nmol) and increasing concentrations of NAD and analysed the products by high-pressure liquid chromatography (HPLC). The peptide that reacted in the absence of NAD gave rise to two peaks (3 and 5), which were analysed by mass spectrometry (Fig. 1b; and Supplementary Information) and correspond to a monomeric (relative molecular mass (M_r) 2370) and a dimeric (M_r 4740) peptide, the latter probably due to oxidation of the peptide at the carboxyl cysteine residue. The same species were observed in reactions with a control bacterial preparation (pET) in the presence of NAD.

The addition of NAD to the reaction containing Sir2 gave rise to three additional peaks (1, 2 and 4) and an alteration in peak 3, which were also analysed by mass spectrometry (Fig. 1c–f; and Supplementary Information). These peaks did not correspond to ADP-ribosylated species, but, rather, to deacetylated species of peptide (Fig. 1g). Peak 4 corresponded to the singly deacetylated dimer (M_r 4698); peak 3 also contained the doubly deacetylated dimer (M_r 4656); peak 2 corresponded to the triply deacetylated dimer (M_r 4614), and peak 1 to the singly deacetylated monomer (M_r 2328).

We estimate that at least 27% of the input peptide was deacetylated...