Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling

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The *Drosophila wingless* gene (*wg*) encodes a protein of the Wnt family and is a critical regulator in many developmental processes[1]. Biochemical studies have indicated that heparan sulphate proteoglycans, consisting of a protein core to which heparan sulphate glycosaminoglycans are attached[2], are important for Wg function[3]. Here we show that, consistent with these findings, the *Drosophila* gene *sulfateless* (*sfl*), which encodes a homologue of vertebrate heparan sulphate *N*-deacetylase/*N*-sulphotransferase (an enzyme needed for the modification of heparan sulphate) is essential for Wg signalling. We have identified the product of *division abnormally delayed* (*dally*), a glycosyl-phosphatidyl inositol (GPI)-linked glypican, as a heparan sulphate proteoglycan molecule involved in Wg signalling. Our results indicate that Dally may act as a co-receptor for Wg, and that Dally, together with *Drosophila* Frizzled 2, modulates both short- and long-range activities of Wg.

Wg signalling is defective in *sugarless* (*sgl*) mutants[4]. *sgl* encodes a *Drosophila* homologue of uridine diphosphoglucose dehydrogenase that is required for the formation of glucuronic acid. Because glucuronic acid is required for the formation of heparan sulphate, chondroitin sulphate and dermatan sulphate, it is unclear which classes of proteoglycans are involved in Wg signalling. In the genetic screen[5] that led to the isolation of *sgl*, we also isolated mutations in a second locus *sulfateless* (*sfl*), that showed a similar segment-polarity cuticle phenotype (Fig. 1b). In *sfl* null embryos, the expression patterns of *wg* and *engrailed* (*en*) are reminiscent of those observed in either *wg* or *hedgehog* (*hh*) null mutants (not shown). To investigate further whether Sfl activity is required in Wg signalling, we analysed the effect of *sfl* mutations on the development of stomatogastric nervous system (SNS) and the second midgut constriction, both of which require Wg but not Hh activity[6]. In *sfl* null embryos, the development of these Wg-mediated processes is perturbed (Fig. 1e, g). Consistent with a role for Sfl in Wg signalling, Wg-dependent processes in the wing imaginal disc also require Sfl activity. Wg is required for dorso–ventral patterning and acts over a short range to control the expression of *neuralized* (*neu*) at the wing margin[1] and in the long range to activate the expression of *distaless* (*dll*)[7,8]. In *sfl*-mutant wing discs, the expression of *neu* is abolished (Fig. 1i), and Dll expression is also markedly reduced (Fig. 1k). Our results indicate that Sfl activity is necessary for Wg signalling during both embryonic and wing-disc development.

A complementary DNA encoding the product of *sfl* was isolated (Fig. 2). A search of the protein sequence databases revealed that the putative protein deduced from the *sfl* cDNA is homologous with heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST)[9], which is required specifically for the modification of heparan sulphate glycosaminoglycans (GAGs) but not chondroitin sulphate and dermatan sulphate GAGs. Together, these results provide genetic evidence that heparan sulphate proteoglycans (HSPGs) are involved in Wg signalling and that HSPGs have non-redundant roles with other classes of proteoglycan in the context of Wg signalling.

Heparan sulphate GAGs are attached to various protein cores to form different HSPGs. Because *Drosophila* frizzled 2 (Dfz2) appears as a distinct band on western blots (S. Cumberledge, personal communication; M. Zeidler and N.P., unpublished), not as the smear that is characteristic of proteoglycans[2], it is unlikely that the receptors for Wg/Wnt encoded by members of the Frizzled (Fz) family are heparan-sulphate-modified proteins. The *Drosophila glypican* homologue *dally* appeared to be an excellent candidate because flies homozygous for hypomorphic *dally* alleles exhibit some wing-margin defects[10], a phenotype similar to partial loss of *wg* activity. To examine the role of *dally* in Wg signalling, we first determined the expression of *dally* messenger RNAs in embryos by *in situ* hybridization (Fig. 3). At early stages *dally* transcripts are uniformly expressed; however, at stage 8, *dally* transcripts are enriched in a segmental repeated pattern in three to four cells anterior to *wg*-expressing cells. Double staining for *Dfz2* mRNA and wg–lacZ shows that the 2–3-cell-wide band of *dally*-expressing cells anterior to *wg*-expressing cells also express *Dfz2*[11] (not shown), indicating that *dally* may be involved in Wg signalling.

Next, we examined the cuticle phenotype of *dally* mutant

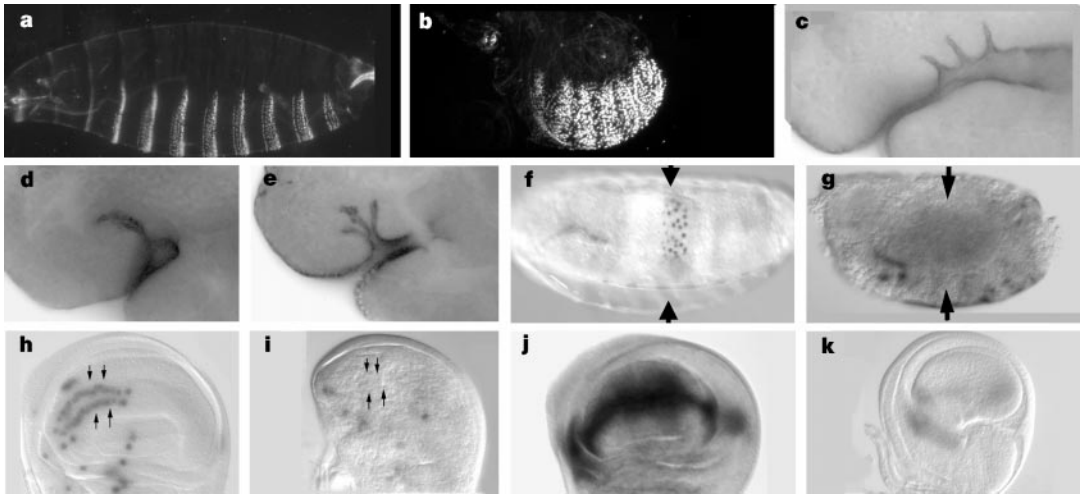

Figure 1 *sfl* is required for Wg signalling. **a**, **b**, Cuticle phenotypes of wild-type (WT) (**a**) and *sfl* (**b**) embryos. **c**–**e**, SNS phenotypes stained by anti-Crumbs antibody in stage 10 embryos of WT (**c**), *wg* (**d**), *sfl* (**e**). As observed for *sgl*[6], the SNS phenotype is similar, although slightly weaker, than in the *wg* mutant (ref. 6). **f**, **g**, The expression of Labial (Lab) in WT (**f**) and *sfl* (**g**) embryos at stage 15; Lab staining, marking the position where the second midgut (arrows) is absent in an *sfl* embryo (**g**). In the wing disc of WT third-instar larvae (**h**), *neuralized* (*neu*)-expressing sensory mother cells visualized using the A101 enhancer trap are found in two rows (arrows), and are missing in $sfl^{l(3)03844}/sfl^{9B4}$ wing disc derived from *sfl* homozygous mutant animals derived from heterozygous mothers (**i**). In WT wing disc (**j**), Dll forms a gradient with its highest level of expression at the dorso–ventral boundary, and is greatly reduced in the $sfl^{l(3)03844}/sfl^{9B4}$ disc (**k**).

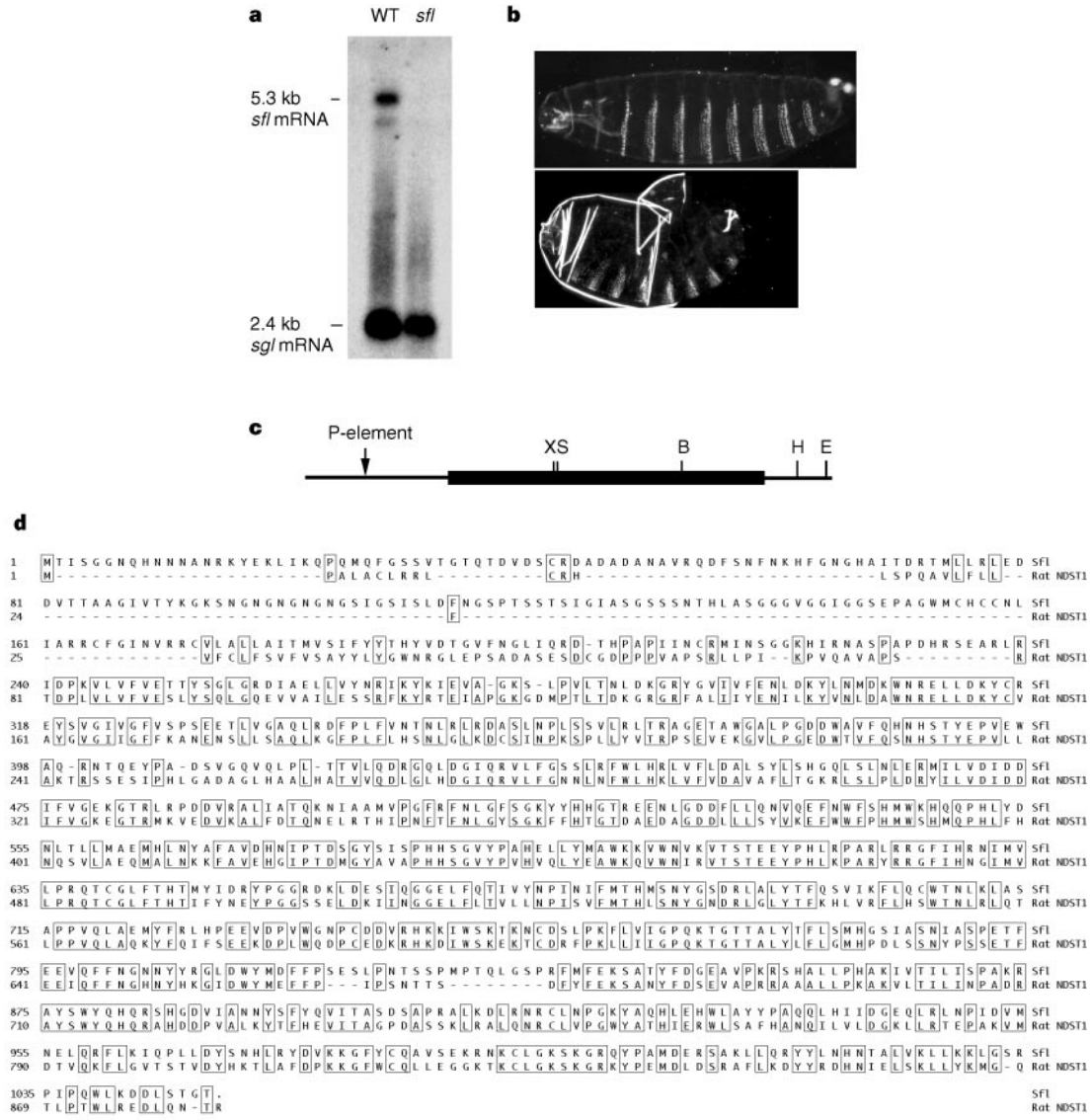

Figure 2 *sfl* encodes heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST). **a**, Northern blot analysis of *sfl* RNA from 0–1.5 h WT and *sfl* mutant embryos. The blot was probed with *sfl* cDNA and *sgl* cDNA[6]. *sgl* was used as an internal control. 5.3 and 2.4 kilobase (kb) mRNAs correspond to the *sfl* and *sgl* transcripts, respectively. **b**, Rescue of *sfl* maternal-effect phenotypes by RNA injection. Top, cuticle phenotype of a paternally rescued *sfl* embryo marked with a *trachealess* (*trh*) mutation that exhibits the defective posterior spiracles[6]. Bottom, cuticle phenotype of *sfl* null embryos derived from GLCs injected with RNA transcribed from the *sfl* full-length cDNA. Of 700 injected embryos derived from females with *trh* GLCs, 120 *sfl* mutant embryos (*trh sfl/sfl*) developed scorable cuticle structures, and 15% of them showed evidence of rescue. **c**, Restriction map of the *sfl* cDNA. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I; X, *Xho*I. The P-element *l(3)03844* is inserted at base 576 of the *sfl* cDNA (686 base pairs (bp) upstream of a putative ATG start codon). The open reading frame encoding Sfl is shown by the thick line. **d**, Putative amino-acid sequence of Sfl protein and comparison with rat NDST1. Identical residues are boxed. The overall identity between Sfl and Rat NDST1 is 51%.

embryos. These mutant embryos exhibit poorly penetrant cuticle segment-polarity defects resembling a partial defect in Wg signalling (Fig. 3d). However, these segmentation defects can be significantly enhanced by removal of one copy of *sfl* in the mother or one copy of *wg* in the embryo (Fig. 3e, f). Because all available *dally* mutations are weak alleles, we used double-stranded RNA (dsRNA) interference to block *dally* gene activity[12]. Embryos injected with *dally* dsRNAs corresponding to the entire coding region of *dally* exhibit severe segment-polarity cuticle defects (Fig. 3g, h), similar to those injected with *wg* or *frizzled* (*fz*) + *Dfz2* dsRNAs[12]. This result, together with the genetic interaction observed between *sfl* and *wg*, strongly supports the proposal that *dally* is a new segment-polarity gene and that it is required for Wg signalling in the embryo. Further, we find that Dally, which migrates as a smear in wild-type extracts, migrates as sharp bands in the protein extracts of *sfl* mutants (Fig. 4). Similarly, Dally is not modified in *sgl* embryos (S. Selleck, personal communication). These results indicate that Dally is a likely substrate of Sgl and Sfl.

To further examine the role of *dally* in Wg signalling, we analysed the function of *dally* during wing-disc development. Consistent with previous reports[10], we found that only 3% of homozygous *dally* animals exhibit wing-margin defects (Fig. 5a). This frequency can be increased 2–3-fold and wing-margin defects are more severe when one copy of *wg* is removed (Fig. 5b). To determine whether Dally cooperates with the Wg receptor Dfz2 in wing patterning, we tested whether *dally* mutations can enhance a loss-of-function *Dfz2* phenotype. When a dominant-negative form of Dfz2 (Dfz2N)[13] is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects (Fig. 5c). However, this phenotype is enhanced in homozygous *dally* mutants (Fig. 5d), indicating that *dally* may potentiate Wg signalling. Furthermore, ectopic expression of a gain-of-function

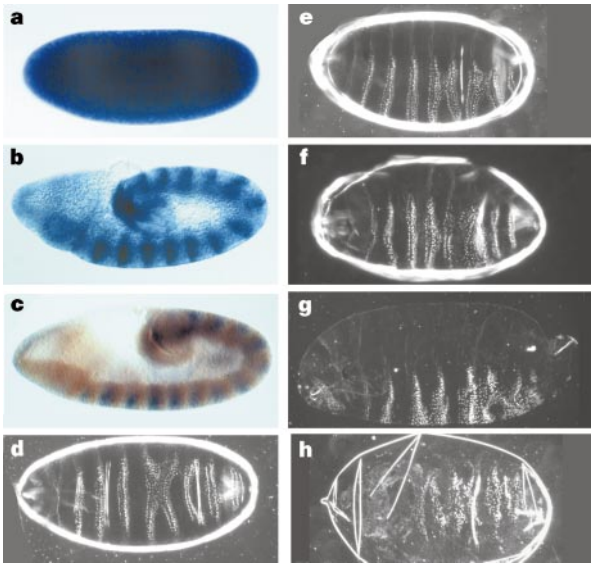

Figure 3 *dally* is a segment-polarity gene. *dally* mRNAs are uniformly distributed in stage 2 embryos (**a**), and are expressed in a segmental repeated pattern at stage 8 (**b**). **c**, *dally* transcripts (blue) are located 3–4 cells anterior to *wg*-expressing cells (brown, *wg–lacZ*). $dally^{P2}$ homozygous embryos derived from females with $dally^{P2}$ or $dally^{\Delta P\text{-}188}$ homozygous GLCs exhibit weak segment-polarity cuticle defects (**d**; 8% penetrance, $n = 760$). More severe defects are detected in $dally^{P2}$ embryos where *sfl* maternal message is eliminated (**e**; 14% penetrance; $n = 780$). Similarly, $dally^{P2}$ homozygous embryos derived from GLCs show a more severe embryonic phenotype if zygotic *wg* is reduced by half (**f**; 16% penetrance, $n = 760$). Embryos injected with *dally* dsRNA develop *wg*-like cuticle defects (**g**, **h**). 48% of the injected embryos ($n = 127$) exhibit defects. Embryos injected with buffer exhibit no cuticle defects ($n = 150$).

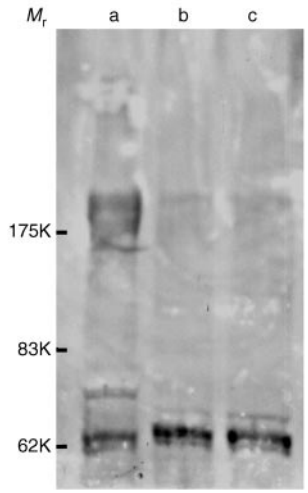

Figure 4 Heparan sulphate GAG modification of Dally in *sfl* mutants. Total proteins from third-instar larvae were analysed by SDS–PAGE followed by western blotting with anti-Dally antibody. In WT larvae (a), Dally migrates as a high relative molecular mass (M_r) smear, characteristic of heparan sulphate-modified Dally, and ~70K unmodified bands. In homozygous $sfl^{l(3)03844}$ (b) or sfl^{9B4} (c) larvae, high M_r heparan sulphate-modified Dally is significantly reduced and sharp bands of unmodified Dally are increased.

Arm protein (Arm^{act}) can fully rescue the wing defects (Fig. 5f), indicating that the enhancement of wing-margin defects in the *dally* mutant is specific to Wg signalling and that Dally acts upstream of Arm. These genetic interactions are consistent with a role for Dally in Wg signalling and indicate that Dally may act with Dfz2 in Wg reception.

If *dally* acts with Dfz2 to transduce Wg signalling, Dally may also

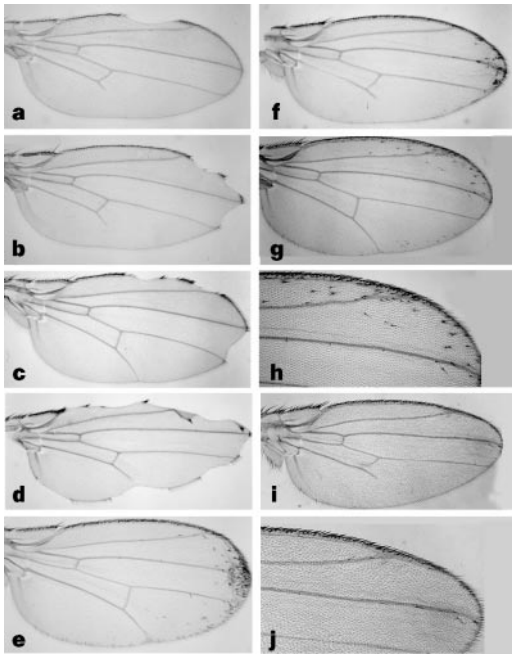

Figure 5 Dally is required for Wg/DFz2 signalling in wing patterning. **a**, 3% of $dally^{P2}$ homozygous flies exhibit a wing notching phenotype ($n = 550$). **b**, Wing phenotype of wg^{IG22}/+; $dally^{P2}/dally^{P2}$ animals is enhanced and shows higher penetrance (8%; $n = 654$). The wing-vein abnormality seen in the *dally* mutant does not seem to involve Wg signalling. **c**, Ectopic expression of Dfz2N at the presumptive wing margin using C96-Gal4 (*UAS-Dfz2N*/+; *C96*/+) is associated with a fully penetrant mild wing-margin defect. **d**, *UAS-Dfz2N*/+; *C96* $dally^{P2}/dally^{P2}$ wing. Decreased *dally* activity strongly enhances the wing defect observed in **c**. **e**, UAS-arm^{act}/+, C96/+ wing. Ectopic expression of arm^{act} results in ectopic bristles on the wing blade. **f**, UAS-arm^{act}/*UAS-Dfz2N*; *C96* $dally^{P2}/dally^{P2}$ wing. Ectopic expression of UAS-arm^{act} fully rescues the margin defect shown in **d**. **g**, **h**, Uniform expression of Dfz2 driven by 69B-Gal4 in *69B-Gal4/UAS-Dfz2* flies leads to wings with ectopic bristles. **i**, **j**, Ectopic bristles are strikingly reduced in the wing of *UAS-Dfz2* $dally^{P2}$/*69BGal4* $dally^{P2}$. **h**, **j**, Higher magnifications of the wings shown in **g** and **i**, respectively.

be required for other functions of Dfz2 in Wg signalling. In the wing blade, Dfz2 is involved in shaping the gradient of Wg distribution and determining the response of cells to Wg[14]. Uniform overexpression of *Dfz2* in the wing pouch leads to ectopic bristle formation in the wing blade, probably reflecting activation of Wg signalling above its normal level. Ectopic expression of *Dfz2* driven by the Gal4 line 69B resulted in wings with ectopic bristles[14] (Fig. 5g, h). In a *dally* mutant background, the formation of ectopic bristles was greatly reduced, indicating that a mutation in *dally* blocks the activity of Dfz2 (Fig. 5i, j).

Our findings indicate that HSPGs have non-redundant roles with other classes of proteoglycan in Wg signalling, and that *dally* encodes a protein core of the HSPGs involved in Wg signalling. There are several possible mechanisms for the function of Dally in Wg signalling. First, Dally could form an active Wg receptor complex with Dfz2. Second, Dally, through its heparan sulphate GAG sequences, could generate a higher-affinity binding site for Wg to Dfz2. Third, as proposed for other co-receptors[15], Dally could limit the free diffusion of Wg by capturing it on the cell surface, thereby increasing its local concentration and the probability that it will interact with less abundant, high-affinity signalling receptors. Biochemical analyses between Dally, Wg and Dfz2 will be required to distinguish between these models. Interestingly, both *Dfz2* and *Fz* encode redundant Wg receptors in the embryo[12]. Thus it is possible that, in addition to having a role in the Wg/Dfz2 interaction, Dally also cooperates with Wg/Fz. Furthermore, Dally regulates the

activity of decapentaplegic (Dpp)[16], a member of the TGF-β superfamily. As we have no evidence for a role for HSPGs in the early function of Dpp in the establishment of dorso–ventral embryonic polarity, the function of Dally may be tissue-specific. Tissue-specific effects of Dally could be generated either through tissue-specific expression of *dally* during development or tissue-specific modification of the heparan sulphate GAG chains linked to the Dally protein core. There is biochemical and genetic evidence to support the model that specific heparan sulphate GAGs decorate the cell surface. In vertebrates, a number of sulphotransferases are differentially expressed in various tissues[17]. In addition, the *Drosophila* gene *pipe*, which is involved in dorso–ventral patterning in the embryo, encodes a putative heparan sulphate 2-*O* sulphotransferase that is expressed in ventral follicle cells[18]. □

Methods

Reagents. The *sfl* alleles are $sfl^{l(3)03844}$ (ref. 5) and sfl^{9B4} (N.P., unpublished). Both $sfl^{l(3)03844}$ and sfl^{9B4} show similar maternal-effect phenotypes. Females with germline clones (GLCs) were generated as described[6]. All the available *dally* alleles are homozygous viable to some extent, with $dally^{P2}$ and $dally^{\Delta P188}$ representing the strongest alleles available[10]. To try to isolate a stronger loss-of-function *dally* allele, we generated a number of new *dally* alleles by P-element excisions. However, none was stronger than the original[10]. Other stocks are: UAS–Dfz2 (ref. 14) and UAS–Dfz2N (ref. 13), UAS–arm^{act} (ref. 19), C96 Gal4 (ref. 20). *dally* cDNA was obtained from S. Selleck[10]. Crumbs, Dll and Lab antibodies were obtained from E. Knust, I. Duncan and T. Kaufman, respectively.

Molecular methods. Molecular characterization of *sfl* and RNA injection were done as described for *sgl*[6]. Western blotting of Dally was performed using a polyclonal Dally antibody, a gift of H. Nakato. The dsRNA synthesis and injection were as described[12].

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The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins

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The regulation of gene expression depends critically upon chromatin structure[1]. Transcription of protein-coding genes can be reconstituted on naked DNA with only the general transcription factors and RNA polymerase II (ref. 2). This minimal system cannot transcribe DNA packaged into chromatin, indicating that accessory factors may facilitate access to DNA. Two classes of accessory factor, ATP-dependent chromatin-remodelling enzymes[3] and histone acetyltransferases[4], facilitate transcription initiation from chromatin templates. FACT (for facilitates chromatin transcription) is a chromatin-specific elongation factor required for transcription of chromatin templates *in vitro*[5,6]. Here we show that FACT comprises a new human homologue of the *Saccharomyces cerevisiae* Spt16/Cdc68 protein and the high-mobility group-1-like protein structure-specific recognition protein-1. Yeast *SPT16/CDC68* is an essential gene that has been

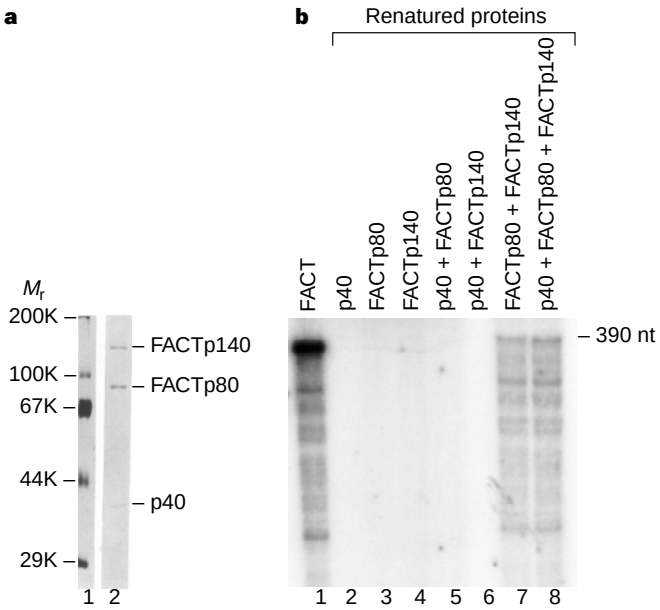

Figure 1 Recovery of FACT activity from renatured p140 and p80 subunits. **a**, Silver-stained gel (phosphocellulose column, fraction 9) showing proteins used in FACT renaturation experiment. The positions of FACTp140, FACTp80, the p40 protein and protein relative molecular mass (M_r) markers are indicated. **b**, Mixtures of renatured polypeptides assayed for FACT activity. Polypeptides were renatured alone and in all possible combinations and were used in transcription reactions on remodelled chromatin templates containing Gal4–VP16 and a reconstituted transcription system (lanes 2–8). Purified FACT was used in lane 1. FACT activity was measured by the appearance of long RNA molecules. The position of the full-length, 390-nucleotide RNA product is indicated.