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An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming

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The priming step of synaptic vesicle exocytosis is thought to require the formation of the SNARE complex, which comprises the proteins synaptobrevin, SNAP-25 and syntaxin[1–3]. In solution syntaxin adopts a default, closed configuration that is incompatible with formation of the SNARE complex[4]. Specifically, the amino terminus of syntaxin binds the SNARE motif and occludes interactions with the other SNARE proteins. The N terminus of syntaxin also binds the presynaptic protein UNC-13 (ref. 5). Studies in mouse, *Drosophila* and *Caenorhabditis elegans* suggest that UNC-13 functions at a post-docking step of exocytosis, most likely during synaptic vesicle priming[6–8]. Therefore, UNC-13 binding to the N terminus of syntaxin may promote the open configuration of syntaxin[9]. To test this model, we engineered mutations into *C. elegans* syntaxin that cause the protein to adopt the open configuration constitutively[4]. Here we demonstrate that the open form of syntaxin can bypass the requirement for UNC-13 in synaptic vesicle priming. Thus, it is likely that UNC-13 primes synaptic vesicles for fusion by promoting the open configuration of syntaxin.

Using NMR spectroscopy, it has been shown that syntaxin adopts a closed configuration in solution[4]. However, mutations in two highly conserved amino acids (L165A, E166A) cause syntaxin to adopt a constitutively open configuration *in vitro*[4]. We made the corresponding mutations in *C. elegans* syntaxin (L166A, E167A; open syntaxin). Similar to vertebrate open syntaxin, the mutated *C. elegans* protein can bind synaptobrevin but not UNC-18 in pull-down assays (data not shown).

Expression of open syntaxin can fully rescue null mutations of syntaxin. *unc-64(js115)* is a null allele of the gene encoding *C. elegans* syntaxin[10]. Homozygotes of *unc-64(js115)* are completely paralysed and arrest development after hatching (Fig. 1a). This developmental defect was fully rescued by expression of wild-type syntaxin or the open form of syntaxin in null mutants (Fig. 1a). Expression of either form of syntaxin rescued the behavioural phenotypes associated with *unc-64(js115)* (Fig. 1b). Furthermore, expression of open syntaxin did not affect neuronal development (data not shown).

To test if open syntaxin could restore synaptic vesicle fusion we quantified whole-cell voltage-clamp currents recorded from the neuromuscular junctions of dissected nematodes[11]. The frequency of endogenous synaptic events in transgenic animals overexpressing wild-type syntaxin or open syntaxin were comparable ($P > 0.05$) to those in wild-type animals (Fig. 1c). In addition, stimulation of the presynaptic nerve cord in animals expressing wild-type or open syntaxin resulted in evoked release amplitudes that were not significantly different ($P > 0.05$) from those of wild-type animals (Fig. 1d). These data demonstrate that open syntaxin can substitute for wild-type syntaxin in basal synaptic transmission.

It was possible that these mutations in syntaxin created a gain-of-function protein that executes vesicle fusion independent of normal synaptic mechanisms. To demonstrate that open syntaxin functioned by means of the normal mechanisms of exocytosis, we confirmed that open syntaxin mediates exocytosis through the SNARE complex and requires calcium influx. We made a double mutant with open syntaxin and the synaptobrevin null allele *snb-1 (js124)*[12]. The larval lethality of *snb-1(js124)* was not rescued by open syntaxin ($n = 25$). To determine if open syntaxin could bypass the requirement of the N-type calcium channel UNC-2, we generated double mutants with *unc-2(e55)*[13]. Similarly, open syntaxin did not suppress the behavioural and neurotransmission defects associated with *unc-2* mutants (Fig. 2). These data demonstrate that open syntaxin functions through the normal synaptic machinery and validates it as a reagent to test the function of UNC-13.

Electrophysiological studies in mouse, *Drosophila* and *C. elegans* indicate that UNC-13 is probably required to prime synaptic vesicles for exocytosis[6–8]. Because UNC-13 binds the N terminus of syntaxin[5], it has been suggested that UNC-13 might promote the open state of syntaxin to enable vesicle priming[9]. If this model were correct then expression of open syntaxin should bypass the requirement of UNC-13 in synaptic vesicle exocytosis. To test this model we expressed the constitutively open form of syntaxin in *unc-13(s69)* animals. *unc-13(s69)* is a frameshift mutation before the highly conserved carboxy terminus of UNC-13 (ref. 14) and causes an almost complete paralysis in mutant nematodes (Fig. 3a).

Overexpression of wild-type syntaxin failed to rescue any of the *unc-13(s69)* behavioural phenotypes including locomotory movements, pharyngeal pumping and defecation cycles (Fig. 3a, b). However, overexpression of open syntaxin in *unc-13(s69)* animals caused a significant ($P < 0.0001$) improvement in all three behaviours (Fig. 3a, b). The suppression of the *unc-13* behavioural phenotypes suggested that open syntaxin was able to bypass the requirement for UNC-13 in synaptic transmission.

As almost no synaptic vesicle fusion is observed in *unc-13(s69)* animals, previous studies could not exclude UNC-13 from having a function in membrane fusion[6–8]. If UNC-13 mediates fusion of the vesicular and plasma membranes, then open syntaxin should not be able to bypass this requirement for UNC-13 in fusion. To directly assay whether synaptic vesicles can fuse with the plasma membrane in the absence of UNC-13, we recorded spontaneous, miniature postsynaptic currents in the absence of calcium. In *unc-13(s69)* animals there was essentially no synaptic vesicle fusion in 0 mM calcium (Fig. 3c). Overexpression of wild-type syntaxin did not rescue vesicle fusion in *unc-13(s69)* animals. However, overexpression of open syntaxin completely restored spontaneous fusion in *unc-13(s69)* animals to wild-type levels. These data demonstrate that UNC-13 is not required at the fusion step during exocytosis.

UNC-13 contains several C2 domains that are calcium-binding motifs[14–16]. The presence of these domains suggested that UNC-13 might be a calcium sensor for synaptic vesicle exocytosis. If UNC-13 were the sole calcium sensor, then in the absence of UNC-13 there should be no calcium-dependent release. Consistent with this hypothesis, *unc-13(s69)* mutants completely lack calcium-dependent evoked responses (Fig. 3e), and overexpression of wild-type syntaxin failed to rescue evoked release in the *unc-13(s69)* mutants. However, overexpression of open syntaxin completely restored

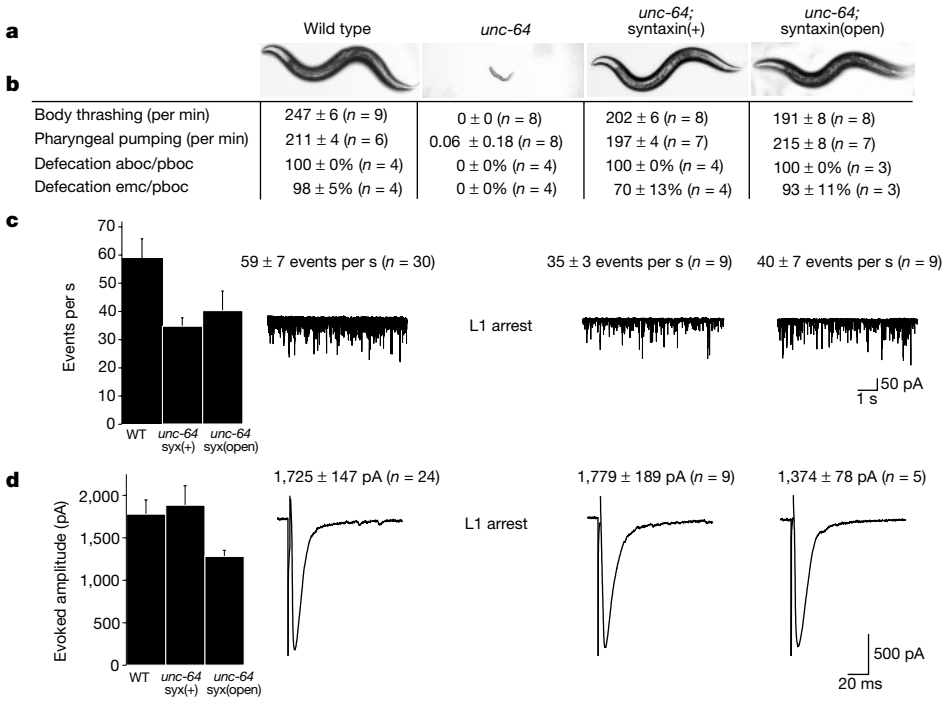

Figure 1 Expression of the constitutively open form of syntaxin rescued the syntaxin null *unc-64(js115)*. **a**, Transformation with either wild-type syntaxin (+) or open syntaxin (open) restored viability to *unc-64(js115)*. **b**, Behavioural phenotypes of *unc-64(js115)* animals overexpressing either wild-type syntaxin or open syntaxin are comparable to wild-type animals. Strains overexpressing either form of syntaxin are slightly sluggish on plates, as reflected in significantly lower thrashing rates ($P < 0.002$). This behavioural deficit is a consequence of syntaxin overexpression and not incomplete rescue, as animals that are wild type for *unc-64* overexpressing either open syntaxin or wild-type syntaxin have the same phenotype (data not shown). aboc, anterior body contraction; pboc, posterior body contraction; emc, enteric muscle contraction. **c**, Endogenous release rates in 5 mM Ca^{2+} saline were restored to wild-type levels ($P > 0.05$) in both *unc-64;* syntaxin(+) and *unc-64;* syntaxin(open) animals. **d**, Depolarization of the ventral nerve cord elicited large evoked post-synaptic currents not significantly different from the wild type ($P > 0.05$) in both *unc-64;* syntaxin(+) and *unc-64;* syntaxin(open) animals. In all figures: left, recordings in 5 mM Ca^{2+} plotted as mean ± s.e.m.; right, sample traces.

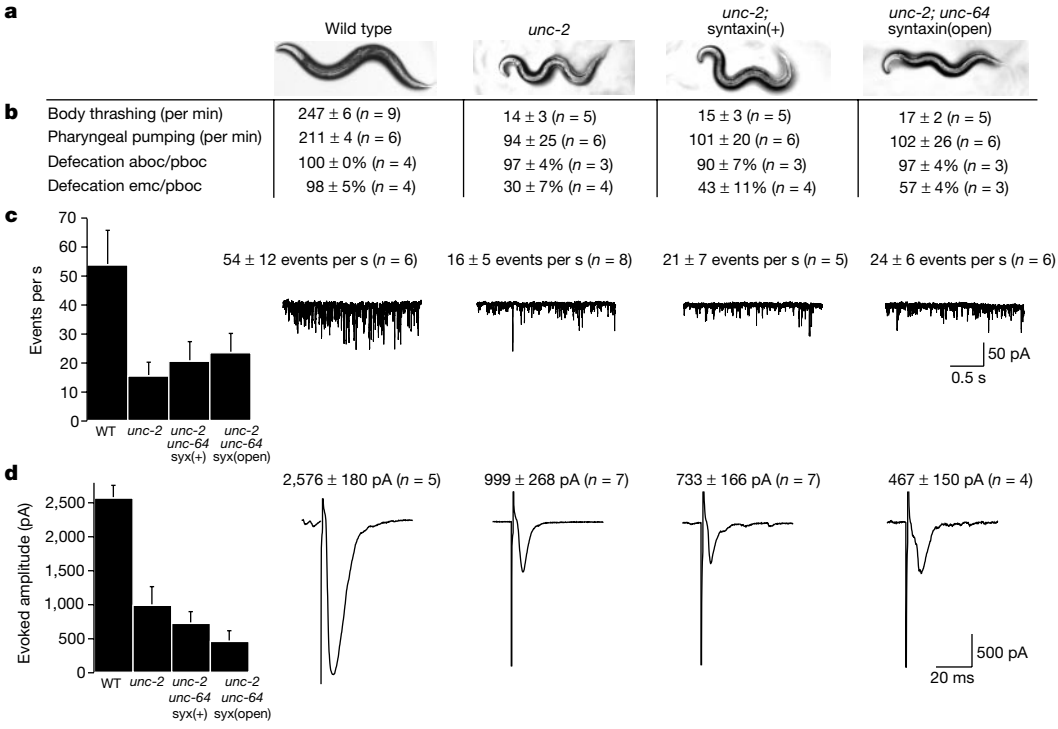

Figure 2 Open syntaxin failed to rescue mutants of the alpha subunit of the N-type voltage-gated calcium channel, UNC-2. **a**, The loss-of-function allele *unc-2(e55)* results in an uncoordinated locomotory phenotype. Expression of either syntaxin(+) or syntaxin(open) failed to restore locomotion to *unc-2(e55)* animals. **b**, Behavioural phenotypes associated with *unc-2* are not suppressed by the overexpression of syntaxin(+) or syntaxin(open). **c**, Loss of the UNC-2 calcium channel results in a significant decrease in endogenous fusion events in 5 mM Ca^{2+} compared with the wild type ($P < 0.005$). Overexpression of syntaxin(+) or syntaxin(open) failed to restore wild-type, calcium-dependent endogenous currents in *unc-2* animals ($P < 0.05$). **d**, Loss of UNC-2 function results in a significant decrease in calcium-dependent evoked response compared with the wild type ($P < 0.002$). Overexpression of wild-type or open syntaxin failed to rescue the evoked response in *unc-2* animals ($P < 0.0001$).

evoked responses to wild-type levels (Fig. 3e). These normal responses to calcium in the absence of UNC-13 are consistent with the observation that overexpression of rat UNC-13 in chromaffin cells does not affect the calcium sensitivity of release[17]. Together, these data demonstrate that UNC-13 is not the calcium sensor that triggers fusion of synaptic vesicles. Previously we demonstrated that UNC-13 is essential for a post-docking step in synaptic vesicle exocytosis[8]. Similar results were obtained in null alleles of the *Drosophila* homologue, Dunc13 (ref. 7) and the mouse homologue, Munc13-1 (ref. 6). These studies concluded that UNC-13 probably has a role in priming of synaptic vesicles for fusion but they could not exclude a role for UNC-13 in either calcium sensing or in the fusion event itself. Our data demonstrate that UNC-13 is not required for calcium sensing or for fusion and strongly suggest a highly specific role for UNC-13 in the priming step of synaptic vesicle exocytosis.

Vesicles become fusion competent at the priming step of exocytosis. At the molecular level, priming is thought to be mediated by the formation of the SNARE complex[1–3]. Overexpression of Munc13-1 in bovine chromaffin cells accelerates the forward rate constant for the priming of morphologically docked, large dense-core vesicles without affecting the rate of fusion or the calcium sensitivity of release[17]. This stage of dense-core vesicle exocytosis coincides with the association of the SNARE proteins[18]. Although Munc13-1 levels are normally very low in chromaffin cells, these observations suggest that UNC-13 can function to promote dense-core vesicle priming, possibly by promoting formation of the SNARE complex. Our data confirm and extend these studies by demonstrating that UNC-13 promotes the priming of synaptic vesicles by acting through syntaxin. Specifically, the role of UNC-13 may be to bind the autoinhibitory domain of syntaxin to promote or maintain the open state and thus facilitate formation of the SNARE complex.

The rescue of the Unc-13 phenotype from no evoked responses to wild-type levels of evoked responses by open syntaxin is a dramatic result; however, these animals are not completely wild type. First, body thrashing and locomotory activity of the mutant is greatly reduced compared with the wild type (Fig. 3b). Second, measures of endogenous release of synaptic vesicles in the presence of calcium is also greatly reduced compared with the wild type (Fig. 3d). There are several possible explanations for these results. One potential explanation is that the L166A and E167A mutations do not completely mimic the conformation of syntaxin when it is bound to UNC-13. Alternatively, UNC-13 may have an additional role in vesicle exocytosis, possibly to tether synaptic vesicles near calcium channels. Nevertheless, these data suggest that UNC-13 stimulates priming by opening syntaxin either through the direct interaction previously demonstrated or by acting on another protein, such as UNC-18. □

Methods

Genetics

We grew strains on nematode growth media (NGM)[19]. The wild-type strain is N2 Bristol. The *unc-13(s69)* allele is a putative null and contains a 5-base-pair deletion, which creates a frameshift at residue 1,029 (ref. 14). The syntaxin *unc-64(js115)* allele contains a nonsense mutation at codon 71 (ref. 10). In all cases the syntaxin(+) strain is EG1986 *unc-64*

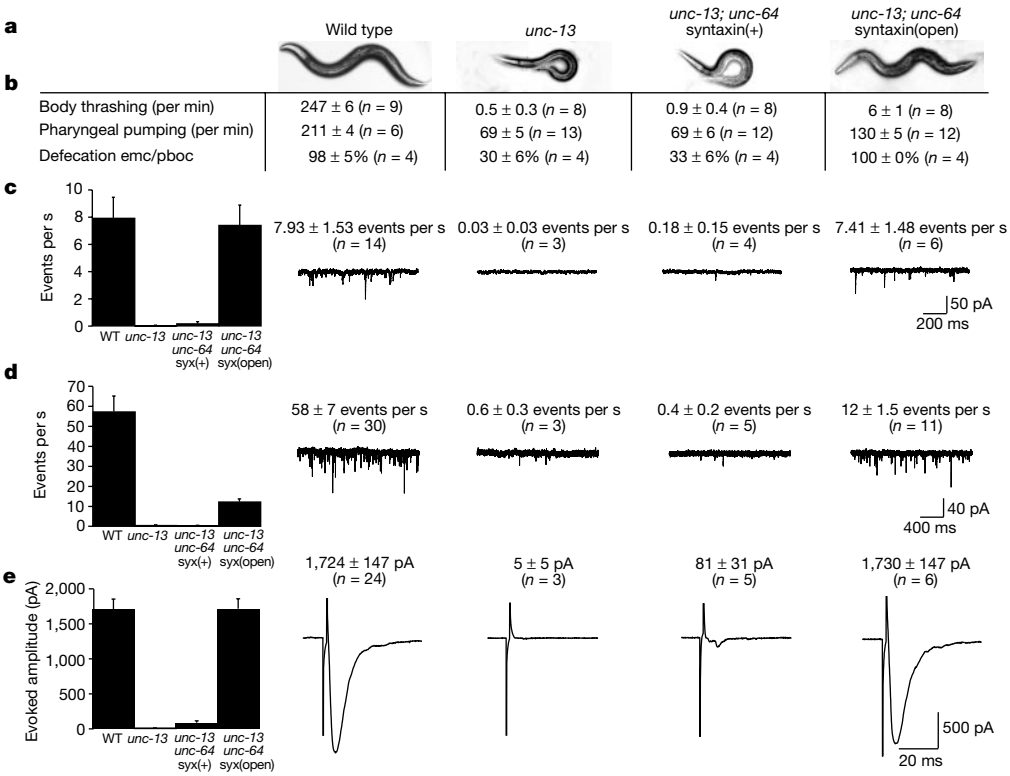

Figure 3 The open form of syntaxin rescues the *unc-13(s69)* mutant. **a**, Overexpression of open syntaxin, but not wild-type syntaxin, partially suppresses *unc-13* paralysis. **b**, Expression of the open form of syntaxin significantly suppresses the body thrashing ($P < 0.0005$), pharyngeal pumping ($P < 0.0001$) and the defecation defects ($P < 0.0001$) associated with *unc-13(s69)*. **c**, Synaptic vesicle fusion can occur in the absence of UNC-13. The average spontaneous event frequency in 0 mM $Ca^{2+}$of *unc-13(s69);* syntaxin(open) animals was not significantly different ($P > 0.8$) from that of the wild type. **d**, Expression of open syntaxin partially restores endogenous vesicle fusion events in *unc-13(s69)* animals. The endogenous event frequency recorded in 5 mM Ca^{2+} was significantly greater in *unc-13(s69);* syntaxin(open) than in both *unc-13(s69)* ($P < 0.005$) *or unc-13(s69);* syntaxin animals ($P < 0.0005$), but significantly lower than wild-type levels ($P < 0.0001$). **e**, Open syntaxin rescues calcium-dependent evoked responses in 5 mM Ca^{2+} in the *unc-13(s69)* mutant. Ventral cord stimulation elicited robust currents at the neuromuscular junctions of *unc-13(s69);* syntaxin(open) animals similar to those in the wild type. The average amplitude of the evoked responses in *unc-13(s69);* syntaxin(open) animals was not statistically different ($P > 0.05$) from that of wild-type animals.

(js115); oxIs33[*unc-64(+); Punc-122*::GFP] I. The syntaxin(open) strain is EG1985 *unc-64 (js115) oxIs34*[*unc-64*(L166A/E167A); *Pmyo-2*::GFP] III. We generated the syntaxin(+) strain by microinjecting pTX21, a subclone of the *unc-64* locus[10], into the germline of NM979 hermaphrodites (genotype: *unc-64(js115)/bli-5(e518)*) along with plasmid pPD97/98(*Punc-122*::GFP)[20] as a dominant co-injection marker. Green fluorescent protein (GFP+) transgenic progeny were assayed for rescue of *unc-64(js115)* segregants. Three out of three lines carrying pTX21 were fully rescued. Stably transmitted rescuing arrays were integrated into the genome by X-ray irradiation. The syntaxin(open) strain was generated in a similar fashion by microinjecting pJR04, a variant of pTX21 containing the corresponding L166A/E167A mutations in the *unc-64* coding region, along with plasmid pPD118.33 (*Pmyo-2*::GFP) (1997 Fire vector kit) as the dominant co-injection marker. Two out of two lines carrying pJR04 were fully rescued. The *unc-13(s69);* syntaxin(+) strain is EG1983 *unc-13(s69) oxIs33; unc-64(js115)*. The *unc-13(s69);* syntaxin(open) strain is EG1984 *unc-13(s69); unc-64(js115) oxIs34*. The *snb-1* null allele is *js124* (ref. 12) and the *unc-2* loss-of-function allele is *e55* (ref. 19).

Molecular biology

Plasmid pJR04: *unc-64*(L166A/E167A) was generated from pTX21 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and oligonucleotides oJR005 and oJR006. The sequence of pJR04 was confirmed using an Applied Biosystems automated DNA sequencer at the Sequencing Core Facility (University of Utah). Sequence of oligonucleotides: oJR005, ggagatgaggatgcggccgaaatgattgagagcgg; oJR006, ccgctctcaatcattt cggccgcatcctcatctcc.

Behavioural assays

Body thrashing, pharyngeal pumping in the presence of food and the defecation cycle were assayed as described[21,22]. Overexpression of wild-type or open syntaxin in *unc-64(js115)* resulted in sluggish animals. This subtle phenotype was also observed in *unc-64(+)* strains overexpressing either wild-type or open syntaxin. Therefore, sluggish movement is due to syntaxin overexpression rather than incomplete rescue. Furthermore, several transgenic arrays and many integrants showed similar behaviours, thus the behavioural defects are not due to the specific composition of the array or the site of integration (data not shown).

Electrophysiology and worm dissection

We performed electrophysiological methods as described[8,11]. Briefly, animals were immobilized with a cyanoacrylic glue and a lateral incision was made to expose the ventral medial body wall muscles. Electrophysiological recordings from muscles were made in the whole-cell voltage-clamp configuration (holding potential −60 mV) at room temperature (21 °C) using an EPC-9 patch-clamp amplifier (HEKA) and digitized at 2.9 kHz through an ITC-16 interface (Instrutech). Data were acquired by Pulse software (HEKA) run on a Power Mac 6500/225. The bath solution contained 150 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 15 mM HEPES, pH 7.35, sucrose to ~330 mosmol. The pipette solution contained: 120 mM KCl, 20 mM KOH, 4 mM $MgCl_2$, 5 mM *N*-tris (hydroxymethyl)methyl-2- aminoethane-sulphonic acid, 0.25 mM $CaCl_2$, 4 mM NaATP, 36 mM sucrose, 5 mM EGTA, pH 7.2, sucrose to 315 mosmol. Subsequent analysis and graphing were performed using Pulsefit (HEKA), Mini Analysis (Jaejin Software) and Igor Pro (Wavemetrics). All statistically derived values are given as mean ± s.e.m.

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Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas

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Genomic instability promotes tumorigenesis and can occur through various mechanisms, including defective segregation of chromosomes or inactivation of DNA mismatch repair[1]. Although B-cell lymphomas are associated with chromosomal translocations that deregulate oncogene expression[2], a mechanism for genome-wide instability during lymphomagenesis has not been described. During B-cell development, the immunoglobulin variable (V) region genes are subject to somatic hypermutation in germinal-centre B cells[3]. Here we report that an aberrant hypermutation activity targets multiple loci, including the proto-oncogenes *PIM1*, *MYC*, *RhoH/TTF* (*ARHH*) and *PAX5*, in more than 50% of diffuse large-cell lymphomas (DLCLs), which are tumours derived from germinal centres. Mutations are distributed in the 5′ untranslated or coding sequences, are independent of chromosomal translocations, and share features typical of V-region-associated somatic hypermutation. In contrast to mutations in V regions, however, these mutations are not detectable in normal germinal-centre B cells or in other germinal-centre-derived lymphomas, suggesting a DLCL-associated malfunction of somatic hypermutation. Intriguingly, the four hypermutable genes are susceptible to chromosomal translocations in the same region, consistent with a role for hypermutation in generating translocations by DNA double-strand breaks[4–6]. By mutating multiple genes, and possibly by favouring chromosomal translocations, aberrant hypermutation may represent the major contributor to lymphomagenesis.

Somatic hypermutation occurs in germinal-centre B cells and is found in all germinal-centre-derived B-cell tumours[3]. This process