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JOINTLESS is a MADS-box gene controlling tomato flower abscission zone development

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Abscission is a universal and dynamic process in plants whereby organs such as leaves, flowers and fruit are shed, both during normal development, and in response to tissue damage and stress[1]. Shedding occurs by separation of cells in anatomically distinct regions of the plant, called abscission zones (AZs). During abscission, the plant hormone ethylene stimulates cells to produce enzymes that degrade the middle lamella between cells in the AZ. The physiology and regulation of abscission at fully developed AZs is well known[2,3], but the molecular biology underlying their development is not. Here we report the first isolation of a gene directly involved in the development of a functional plant AZ. Tomato plants with the *jointless* mutation[4] fail to develop AZs on their pedicels and so abscission of flowers or fruit does not occur normally. We identify *JOINTLESS* as a new MADS-box gene in a distinct phylogenetic clade separate from those functioning in floral organs. We propose that a deletion in *JOINTLESS* accounts for the failure of activation of pedicel AZ development in *jointless* tomato plants.

In tomato, the pedicel AZ, an indented region consisting of a band of anatomically distinct cells, is located in the midpoint of the pedicel[5]. The tomato mutation *jointless* (*j*; ref. 4) completely suppresses the formation of pedicel AZs. In addition, *jointless* affects determinate growth; the inflorescence meristems revert to vegetative growth after forming only one or two flowers, resulting in a 'leafy' inflorescence phenotype[6–8]. *Jointless* has agronomic value and is widely used in the processing tomato industry. The lack of AZs on jointless pedicels yields 'stemless' tomato fruit, which aids mechanical harvesting and prevents physical wounding during transportation. *Jointless* is a simple recessive mutation and is genetically mapped to chromosome 11 (refs 4, 9).

We previously mapped *jointless* to a 3.0 cM interval between restriction-fragment length polymorphism (RFLP) markers TG523 and RPD158 (Fig. 1a; ref. 10). To isolate *jointless*, we constructed a yeast artificial chromosome (YAC) contig encompassing the locus and showed that two YAC ends, TY159L and TY143R, co-segregate genetically with *jointless*[11]. *Jointless* is contained within 100 kilobases (kb) of TG523, on the basis of a physical to genetic distance ratio of 86 kb/cM. To isolate candidate genes, we used TG523 to screen a wild-type tomato BAC library[12]. We found that a 120-kb bacterial artificial chromosome (BAC) clone (240K4) contains TG523 and the *jointless*-co-segregating YAC end TY159L (Fig. 1a). As the size of 240K4 was much larger than the estimated physical distance from TG523 to TG159L/*jointless* (~86 kb), this BAC probably contained the wild-type *jointless* gene. Therefore we shot-gun sequenced and annotated it (L.M., D.B., M.A.B. and R.A.W., unpublished data). We detected 13 open reading frames (ORFs) from 240K4 and evaluated them by sequence similarity searches in GenBank. One ORF, 240K4.12, located within ~30 kb of TY159L, has significant protein sequence similarity to the conserved domains of MADS-box genes, which are important in plant development[13–15]. We named this ORF *LeMADS* for *Lycopersicon esculentum* MADS-box gene, and investigated it in detail.

To establish a correlation between *LeMADS* and *jointless*, we used a polymerase chain reaction (PCR) product containing the MADS region as a probe to hybridize DNA from the *jointless* near isogenic lines (NILs) LA3021 (wild type) and LA3023 (*jointless*). All five restriction enzymes tested revealed polymorphism between these two NILs (data not shown), indicating that there could be a deletion in the region. We amplified the putative deletion region by PCR and sequenced it. There is a 939-base-pair (bp) deletion in the *jointless* allele in the 5′ region of *LeMADS*, including the first 33 bp of the MADS domain (Fig. 1b). As *jointless* is a spontaneous mutation[4], the correlation of DNA polymorphism between NILs in a gene co-segregating with the *jointless* phenotype indicated that *LeMADS* was a strong candidate for *JOINTLESS*.

For complementation and antisense suppression experiments, we amplified the *LeMADS* complementary DNA from a wild-type tomato cDNA library by PCR and cloned it in both sense and antisense orientations into the binary vector pBI121 behind the CaMV 35S promoter. 5′ and 3′ rapid amplification of cDNA end (RACE) experiments showed that the cDNA sequence obtained from the cDNA library was full length at 1,010 bp. We used the sense

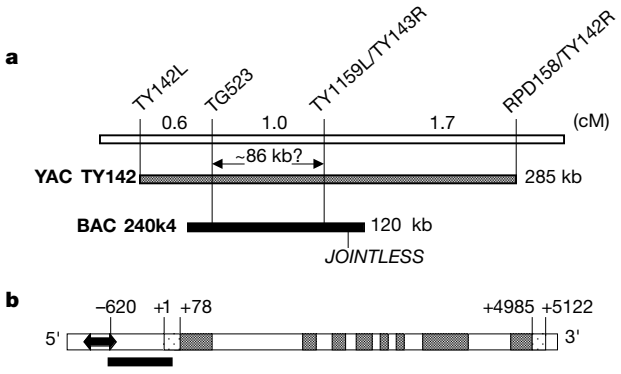

Figure 1 The tomato *JOINTLESS* gene. **a**, Genetic map of the locus on chromosome 11. The TY142L and TY142R are ends of TY142. *Jointless* co-segregates with two other YAC ends TY159L and TY143R. The BAC clone 240k4 contains the flanking genetic marker TG523 and YAC end TY159L. **b**, Diagram of the *JOINTLESS* gene. Black arrow, inverted repeats; +1, transcription start point; dense-dotted boxes, exons; light-dotted boxes, 5′ and 3′ untranslated regions; solid bar, deleted region in *jointless* allele.

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construct to transform *jointless* tomato LA3023 for mutant rescue, and tested the antisense construct for its ability to suppress AZ development in wild-type tomato (LA3021).

We obtained 17 sense and 7 antisense transgenic plants. Among the *jointless* tomato plants (LA3023) transformed with the sense construct, the phenotype of 2 out of 17 transgenic plants appeared normal, with AZ-like structures on most flower pedicels (Fig. 2b). After prolonged growth in the greenhouse, eight additional sense transgenic plants developed AZ-like structures on their pedicels. However, the position of restored AZs on the pedicels of transgenic plants varied. In sense transgenic plants, the phenocopied AZs usually developed closer to or even at the base of a flower (Fig. 2b and c, flowers 6–8), rather than at the midpoint of the pedicels as in the wild type. The rescued AZs appeared normal (Fig. 2c, flowers 1, 4, 7 and 8) and were the breakpoints on the pedicels when the flowers died or the fruits ripened. For the remaining sense-transformed plants, we often observed severe bending at the point of presumed AZs (Fig. 2c, flowers 1 and 3). The two plants with nearly perfect restoration of the AZs contained 1–2 copies of the transgene whereas the remaining had more than 2 copies, as shown by Southern analysis. We speculate that the overexpression of *LeMADS* in the transgenic plants with multi-copy sense transgenes caused co-suppression of the expression of *LeMADS*.

Among the wild-type plants (LA3021), transformed with the antisense construct, six out of seven either partially or completely failed to develop AZs on their flower pedicels. We often observed AZ-like bumps in partially suppressed flower pedicels (Fig. 2e). Southern analysis showed that the antisense construct in the seventh plant, whose pedicels looked normal, was rearranged. Unexpectedly, we observed reversions of antisense-suppressed pedicels (Fig. 2f, flowers 1–3) to pedicels with bumps (Fig. 2f, flowers 4 and 6) or AZ-like structures (Fig. 2f, flower 5) in a single inflorescence. The reason for such a reversion to AZ formation is unknown.

To confirm that *LeMADS* was the *JOINTLESS* gene, T1 plants from two antisense and two sense primary transgenic lines were examined. Progeny from both sense and antisense transgenic plants that bore multi-copy transgenes showed *jointless* phenotypes (co-suppression in the case of sense plants). In contrast, as expected, progeny from the low-copy antisense transgenic T0 plant containing the transgene showed a *jointless* phenotype, whereas those with no transgene, because of genetic segregation, were wild type (Fig. 3a). Similarly, progeny derived from the low-copy sense-rescued primary transgenic plant that inherited the transgene had AZs, but progeny that did not inherit the transgene were *jointless*. The consistent correlation between the *LeMADS* transgene and the *jointless* phenotype shows that *LeMADS* is *JOINTLESS*.

We compared the cDNA sequence of *JOINTLESS* with the genomic sequence from BAC clone 240K4. The genomic DNA of *JOINTLESS* is approximately 6-kb long with eight exons and seven introns (Fig. 1b). There are two inverted repeats in the 5′ region that are possibly transposon-like elements responsible for the deletion in the *jointless* allele. This deletion completely eliminated the first exon as the other two potential start codons in this exon are followed by stop codons a few base pairs later. Nevertheless, northern analysis using the 3′ sequence of *JOINTLESS* cDNA as a probe shows that gene expression could be detected in *jointless* tomato plants. The major transcript in *jointless* plants is slightly shorter than the wild-type transcript (Fig. 3b). 3′ RACE experiments showed no difference between the two transcripts in the 3′ end. However, 5′ RACE resulted in two bands from both LA3021 and LA3023 (data not shown). Sequencing of the 5′ RACE products indicated that the longer band in wild type is part of the normal cDNA. The longer band in *jointless* has a deletion of 115 bp that was replaced with 102 bp of sequence immediately after the deletion point, plus an additional 20 bp reminiscent sequence from the inverted repeat (data not shown). The replacement produced a seven-base larger, aberrant transcript in *jointless* tomato. The smaller 5′ RACE products were possibly derived from a secondary transcription start point.

In *jointless* tomato, it appeared that the shorter transcript rather than the aberrant longer transcript accumulated (Fig. 3b). In wild-type tomato, we detected *JOINTLESS* RNA in almost all the tissues. The amount of messenger RNA is highest in shoot tips and axillary buds, where pedicels and inflorescences are developing. However,

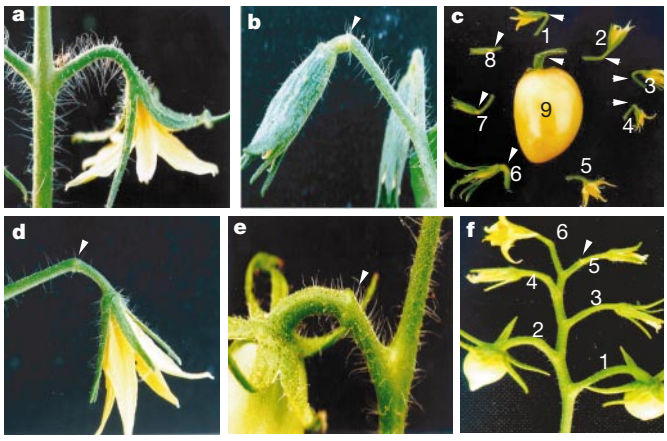

Figure 2 Sense complementation and antisense suppression of AZs in *jointless* and wild-type tomatoes. **a**, A flower from the *jointless* control (LA3023). **b**, A flower with a rescued AZ on its pedicel from a sense construct transformed *jointless* plant. Note that the position of AZ on the pedicel is closer to the flower than those on the wild-type pedicel (Fig. 2d). **c**, Various flowers/fruit from sense-rescued *jointless* plants. Note that flower number 5 is a *jointless* control. **d**, A wild-type flower from jointed control (LA3021). **e**, A pedicel with AZ development partially suppressed by the antisense transgene. The bump-like structure is supposed to be at the position of original AZ in wild-type tomato. **f**, The gradual reversion of suppressed AZs on the same inflorescence from an antisense-transformed wild-type tomato plant from 1 to 6. Arrowheads show the position of the AZ or AZ-like structure.

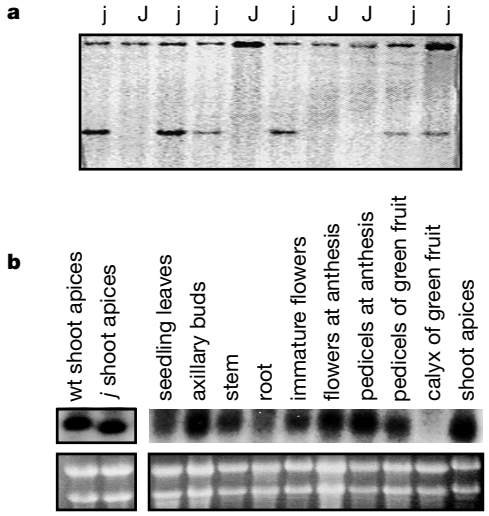

Figure 3 The segregation of transgenes in T1 progeny from an antisense primary transgenic plant (**a**) and expression analysis of *JOINTLESS* gene (**b**). **a**, *Eco*RI and *Hin*dIII double-digested genomic DNA was probed with *JOINTLESS* MADS domain sequence. The top DNA fragments (about 9.0 kb) are the endogenous genomic DNA containing *JOINTLESS*, and the 1.9 kb fragments below are released cDNA sequence plus the 35S promoter from the transgene. J, wild-type pedicel; j, *jointless*. **b**, mRNA from various tissues of wild-type (wt) tomato and *jointless* tomato shoot tips were hybridized with the 3′ sequence of *JOINTLESS*. Note: in the left panel the *jointless* transcript is slightly smaller than that of wild type; tissues in the right panel were all from wild-type tomato.

we also found relatively high amounts of mRNA in fully developed pedicels and flowers (Fig. 3b). The ubiquity of *JOINTLESS* RNA may indicate that the specificity of *JOINTLESS* on pedicel AZ development is regulated at the post-transcriptional level.

A protein sequence search of GenBank revealed that *JOINTLESS* has profound homology with other MADS-box genes. The MADS-box gene most similar to *JOINTLESS* was derived from the *Arabidopsis* Genome Initiative as an annotated ORF from BAC F14M13 (GenBank accession number AC006592). Although there are several MADS-box genes in tomato, they are not among the 20 sequences most similar to *JOINTLESS*, indicating that *JOINTLESS* represents a new kind of MADS-box gene. We developed a phylogenetic tree for *JOINTLESS* with 16 other MADS-box genes from GenBank having the highest matching score (Fig. 4). *JOINTLESS* falls in a distinct group separate from MADS-box genes that are involved in flower development, such as *Arabidopsis* floral homeotic genes *APETALA1* and *CAULIFLOWER*. None of the members inside the clade with *JOINTLESS* are associated with any known phenotypes, though a few, such as stmads 11 and 16 from potato, are expressed mainly in the vegetative tissues[16].

MADS-box genes form a large gene family. They were discovered first in model plants such as *Arabidopsis* and *Antirrhinum*[13–15], and homologues have been found in other plants such as *Brassica*[17], rice[18], potato[16] and tomato[19,20]. Many MADS-box genes are important in the development of plant organs such as floral organs. *JOINTLESS* has a central role in coordinating gene expression underlying the differentiation of the pedicel AZ, and we conclude that it encodes a transcriptional regulator. It is noteworthy that MADS-box genes, such as *AGL8*/*FUL*(ref. 21) and *SHATTERPROOFs* (ref. 22), are involved in *Arabidopsis* fruit dehiscence zone differentiation. Dehiscence is a process in crucifers with common characteristics to tomato fruit abscission[23]. Whether these genes and *JOINTLESS* have similar targets remains unclear. Furthermore, the *jointless* mutation affects a second important process in plant development, namely maintenance of the inflorescence meristem state. Whether *JOINTLESS* or a second, very closely linked gene has this function is not clear, as LA3021 and LA3023 are also mutant for *self-pruning*, which is epistatic to the leafy inflorescence phenotype of *jointless*[8]. Regardless of the role of *JOINTLESS* in regulating meristem fate, the molecular characterization of *JOINTLESS* will provide important insights into the genetic system that controls the differentiation of an anatomically complex and physiologically important structure, the abscission zone. □

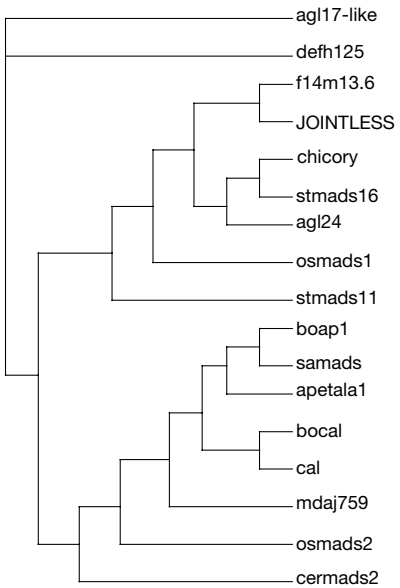

Figure 4 The phylogenetic relationship of *JOINTLESS* with other MADS-box genes. The whole protein sequence of each MADS-box gene was used in the generation of phylogenetic tree. The GenBank accession numbers and brief description of each sequence are agl17-like, CAB37534, *Arabidopsis AGL17*-like protein; defh125, CAA71739, snapdragon; f14m13.6, C006592_22, *Arabidopsis* BAC F14M13; chicory, AAC84133; stmads16, AAB94005, potato; *agl24*, AAC63139, *Arabidopsis*; osmads1, BAA81880, rice; stmads11, AAC63139, potato; boap1, Z37968, a cauliflower a*petala-1/squamosa* homologue; samads, Q41276, white mustard; apetala1, *Arabidopsis AGL7*; bocal, AAA64790, *Brassica oleracea CALIFLOWER*; cal, AAA64789, *Arabidopsis CALIFLOWER*; mdmads, CAA04321, apple tree; osmads2, BAA81883, rice; cermads2, BAA25246, fern (*Ceratopteris richardii*).

Methods

Sequence analysis

ORFs from BAC 240k4 were searched against GenBank using BlastX[24] through the National Center for Biotechnology Information (NCBI) webpage. After cloning the full-length *LeMADS* cDNA, the *LeMADS* protein sequence was deduced. Other MADS-box gene sequences were downloaded from GenBank. Genetics Computer Group (GCG, version 10, Wisconsin Package) program PAUPSEARCH was used for making the phylogenetic tree. Bootstrap analysis using neighbour-joining distance was adopted using the default bootstrap replicates number of 100.

Southern, northern and RACE assays

The MADS conserved region, used as a probe in Southern analysis, was selected according to the genomic sequence and PCR amplified using primers MAOS-1 (5′-CAT TCT CCT CAA TCA TGA CTA A-3′) and MAOS-2 (5′-GGT TTA TTC TTT GTT CCC TC-3′). The PCR product was gel purified using a QIAEX II kit (Qiagen) and labelled with ^{32}P using a DECAprimer II kit (Ambion) for Southern hybridizations. Tomato genomic DNAs were prepared as described[25].

Total RNA from LA3021 and LA3023 was prepared from young leaves using RNeasy Plant Mini Kit (Qiagen). 5′ RACE was performed following the manufacturer's instructions (5′ RACE System, Gibco-BRL, Reagent Assembly v2.0). The three gene-specific primers (GSPs) used were MAOS-CD5 (GSP1), 5′-CTC CCC TCA TTT GCC TTA ATC G-3′; MAOS-CD6 (GSP2), 5′-CTG AAG TTC AAG TGA TGG TTG ATC C-3′; MAOS-CD1r (GSP3), 5′-GCA ACA TCA GCA TCA CAG AGA AC-3′. For 3′ RACE, a polyT anchor primer (5′-GGC CAC GCG TCG ACT AGT AC $T_{(22)}$-3′) was designed with a similar anchor sequence to that provided with the 5′ RACE system. The three GSPs were MAOS-CD1 (GSP1), 5′-GTT CTC TGT GAT GCT GAT GTT GC-3′; MAOS-CD4 (GSP2), 5′-GAA ACA AAT TCT TGA GAG GCG TG-3′; MAOS-CD2r (GSP3), 5′-CA ACA ATT GGA GAG ATC TCT TG-3′.

To avoid cross-hybridization of the MADS-conserved region with other MADS-box genes in tomato, the 3′ region of *LeMADS* was amplified using primers MAOS-CD3 (5′-CTC TTG AAA CTG GAT TGA GCC G-3′) and MAOS-P3Xba as a probe in northern analysis.

Analysis of the deletion in the *jointless* allele

To determine the approximate region of the deletion in the *jointless* allele, the restriction sites derived from computer analysis of the genomic DNA sequence were compared with Southern data. Nested PCR was performed using primers expected to flank the deletion region. Primers MAOS-3 (5′-TGA TGG ATA TGT ACC TTG AGC-3′) and MAOS-4 (5′-GAT AGT TAA AGA TGC GCC TAA C-3′), located about 3.5 kb apart, were used for the first round of PCR. The second round of PCR used primers MAOS-3 and MAOS-5r (5′-GAA TAG ATA TAC ACC CAC ACG-3′). The DNA fragment from LA3023 was cloned into pGEM-T (Promega), and several clones were sequenced on an ABI377 automated DNA sequencer.

cDNA cloning and binary constructs for tomato transformation

To clone a full-length cDNA, primers were designed according to genomic sequence conserved for MADS-box genes and were used together with T7/T3 primers whose sequences were located on the vector used for making the cDNA library, constructed from a wild-type tomato line. PCR reactions were performed directly on phage lysate. By using T3 and MAOS-CD6 (5′-CTG AAG TTC AAG TGA TGG TTG GAT CC-3′), the 78 bp 5′ region was obtained from the cDNA library. A fragment amplified with T7 and MAOS-P5 (5′-CCC TCT TTC TTC ATA ACT CTC TTA G-3′) from the leading region confirmed all the coding sequences. The PCR products were cloned, sequenced and compared with the genomic sequence.

To make sense and antisense constructs, the cDNA sequence containing all the coding region and most of 5′ and 3′ untranslated regions was amplified from the plasmid containing the cloned full-length cDNA. Restriction sites were designed at the 5′ end of the primers, which were referenced with the restriction sites on the binary vector pBI121. The primers used were MAOS-P5Xba (5′-AGT CTC TAG ACC CTC TTT CTT CAT AC TCT CTT AG-3′, underlined is the *Xba*I restriction site), MAOS-P3Sac (5′-GAG TGA GCT CTA CTA ACA ATA ATA TTT ATA TAA AC-3′, underlined is the *Sac*I (*Sst*I) restriction site), and MASO-P5Sac and MAOS-P3Xba with the restriction sites swapped. The PCR products of MAOS-P5Xba/P3Sac were digested with *Xba*I and *Sac*I, gel purified and

cloned into *Xba*I, *Sac*I digested binary vector pBI121 to produce sense cDNA constructs. The PCR products of MAOS-P5Sac/P3Xba were made for antisense constructs in pBI121. The constructs were transformed into *Agrobacterium* strain LBA4404 (Gibco BRL) and used later for tomato transformation[26].

Copy numbers of transgenes in the transgenic plants were estimated by probing their genomic DNA with labelled NPTII gene sequences amplified with NPTII-F, 5′-ACT GGG CAC AAC AGA CAA TCG-3′ and NPTII-R, 5′-AAC GCT ATG TCC TGA TAG CGG-3′. Genomic DNA was digested with *Hin*dIII and/or *Eco*RI where double digestion was designed to release cDNA from the transgenes.

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A mutant of the motor protein kinesin that moves in both directions on microtubules

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Molecular motors move directionally to either the plus or the minus end of microtubules or actin filaments. Kinesin moves towards microtubule plus ends, whereas the kinesin-related Ncd motor moves to the minus ends. The 'neck'—the region between the stalk and motor domain—is required for Ncd to move to microtubule minus ends[1,2], but the mechanism underlying directional motor movement is not understood. Here we show that a single amino-acid change in the Ncd neck causes the motor to reverse directions and move with wild-type velocities towards the plus or minus end; thus, the neck is functional but directionality is defective. Mutation of a motor-core residue that touches the neck residue in crystal structures[2,3] also results in movement in both directions, indicating that directed movement to the minus end requires interactions of the neck and motor core. Low-density laser-trap assays show that a conformational change or working stroke of the Ncd motor is directional and biased towards the minus end, whereas that of the neck mutant occurs in either direction. We conclude that the directional bias of the working stroke is dependent on neck/motor core interactions. Absence of these interactions removes directional constraints and permits movement in either direction.

The Ncd neck mutant that we analysed was based on a *kar3* mutant, *kar3-894*, recovered in a yeast suppressor screen[4]. *kar3-894* has a missense mutation, N378K, in a neck residue that is highly conserved in the carboxy-terminal motor kinesins[5]. We made the corresponding mutation, N340K, in Ncd, and expressed the new

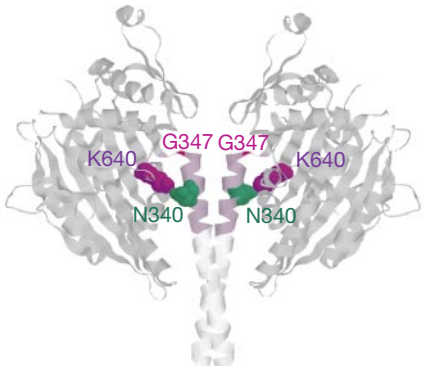

Figure 1 Neck and motor-core mutants. Top, the N340 neck and K640 motor-core residues in a crystal structure[2] of Ncd. The neck residues[1,2], R335–N348 (pink), form a continuous coiled coil with the stalk (white). G347 is near the junction of the neck and motor (grey). The mutants that we analysed are NK11 (N340K), Sup11 (K640N) and NK11 Sup11 (N340K K640N). Bottom, the mutated Ncd neck residue, N340 (green), is conserved in Kar3 and other C-terminal motor kinesins[5]. Residues in the 'a' and 'd' positions (or 1 and 4) of the neck heptad repeat of hydrophobic amino acids are indicated. The motor residues correspond to the first structural element, β1, of the conserved motor core.