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Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease)

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"Lysosomal glycogen storage disease with normal acid maltase", which was originally described by Danon *et al.*[1], is characterized clinically by cardiomyopathy, myopathy and variable mental retardation. The pathological hallmark of the disease is intracytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells. Sarcolemmal proteins and basal lamina are associated with the vacuolar membranes[2,3]. Here we report ten unrelated patients, including one of the patients from the original case report[1], who have primary deficiencies of LAMP-2, a principal lysosomal membrane protein. From these results and the finding that LAMP-2-deficient mice manifest a similar vacuolar cardioskeletal myopathy, we conclude that primary LAMP-2 deficiency is the cause of Danon disease[4]. To our knowledge this is the first example of human cardiopathy–myopathy that is caused by mutations in a lysosomal structural protein rather than an enzymatic protein.

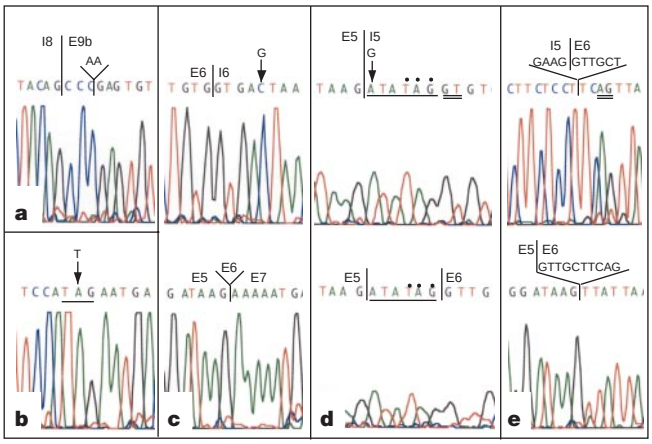

Figure 1 Representative electropherograms of the LAMP-2 gene mutations. **a**, The 2-bp deletion identified in exon 9b of patient 1. **b**, The T-to-A nonsense mutation in exon 4 of patient 2 (stop codon is underlined). **c**, Upper panel, intronic mutation in patient 3; lower panel, sequencing of the RT–PCR product in patient 3, showing exon-6 skipping. **d**, Upper panel, The G-to-A point mutation at the splice-donor site in intron 5 of patient 6; lower panel, sequencing of the RT–PCR product in patient 6, showing a 6-bp insertion at the junction between exons 5 and 6. The inserted sequence (underlined) is derived from the first six nucleotides of intron 5 (underlined), which creates a stop codon (dotted). Most probably, nucleotides 7 and 8, GT (double underlined), in intron 5 are alternatively recognized as a splice-donor site. **e**, Upper panel, the 10-bp deletion in patient 9. This mutation deletes four nucleotides from intron 5 and six from exon 6. Lower panel, sequence of the RT–PCR product in patient 9 reveals the deletion of the 10 nucleotides at the 5′ end of exon 6. The 'AG' (double underlined) in exon 6 immediately after the genomic DNA deletion appears to be recognized as an alternative splice-acceptor site.

In 1981, Danon *et al.*[1] reported two boys with the clinical triad of cardiomyopathy, myopathy and mental retardation. Histochemical and electron microscopic features in the boys' muscle mimicked those of Pompe disease but acid maltase activity was normal. Since then, there have been 14 additional case reports in the English literature. Muscle biopsy is diagnostic and shows a characteristic vacuolar myopathy. The vacuoles are limited by a single membrane, contain various 'debris' including cytoplasmic degradation products and glycogen, and stain intensely for acid phosphatase; all are features of lysosomes. However, the vacuolar membrane occasionally merges with indentations of the sarcoplasmic membrane and stains with antibodies to sarcolemmal proteins such as dystrophin and laminin[2,3]. Moreover, the vacuolar membrane is delineated by basal lamina, which is characteristic of the plasma membrane[3]. Thus, the vacuoles in this disorder share features of both lysosomes and plasma membrane.

Inheritance of Danon disease (DD) has been considered to be X-linked because in most familial cases males are affected predominantly, affected mothers usually have milder and later-onset cardiac symptoms, and no male-to-male transmission has been described. The X chromosome carries the gene encoding lysosome-associated membrane protein-2 (LAMP-2), which structurally consists of a small cytoplasmic tail with a lysosomal membrane targeting signal[5], a transmembrane domain, and a large intraluminal head with two internally homologous domains connected by a hinge region rich in proline, serine or threonine—each domain

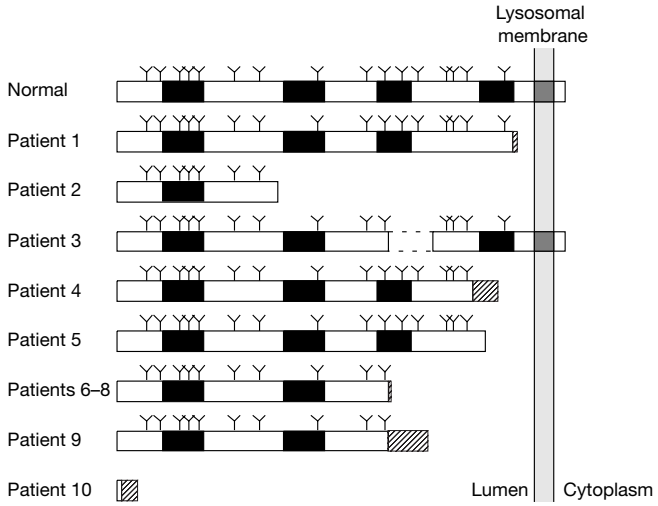

Figure 2 Representation of the mutant LAMP-2b. 'Normal' represents the LAMP-2b amino-acid sequence. Because patient 1, who developed full DD symptoms, has a mutation in exon 9b, the LAMP-2b isoform is most probably responsible for DD. Residue 265, one of the putative disulphide-bond-creating cysteines, and two glycosylation sites are lost by exon-6 skipping in patient 3, which probably results in a significant change in the secondary structure. Black boxes represent the loops created between two cysteine residues flanking each of the four loop regions; Y depicts a glycosylation site; hatched boxes show altered amino acids caused by frameshift mutations; dashes represent missing amino acids due to exon-6 skipping in patient 3. All of the other mutant proteins lack the transmembrane domain (grey bar) because of nonsense or frameshift mutations. Thus, those molecules cannot function as lysosomal 'membrane' proteins.

contains four cysteine residues that form two disulphide bonds. This gene product seemed a particularly promising candidate for the defective protein in DD because 'lysosomes' and 'membranes' are abnormal in this disorder.

The *lamp-2* open reading frame consists of 1,233 nucleotides and encodes 410 amino acids. Exons 1–8 and part of 9 encode a luminal domain; the remainder of exon 9 encodes both a transmembrane domain and a cytoplasmic domain[5]. Human exon 9 exists in two forms, 9a and 9b, which are alternatively spliced and produce two isoforms, LAMP-2a and LAMP-2b, respectively[6]. We sequenced all coding sequences of the LAMP-2 gene in 11 probands and in 54 controls (6 men and 48 women). We also sequenced the exon-flanking sequences in all the samples except for that from patient 10 because of the limited sample availability. We found mutations in all but one proband (Table 1, Fig. 1). We identified eight different mutations: three microdeletions/insertions, two nonsense point mutations, two intronic point mutations and one 10-base-pair (bp) deletion encompassing an exon–intron junction (Fig. 2).

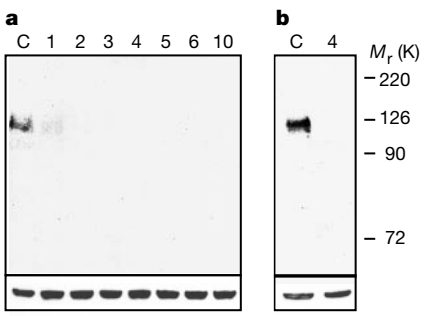

Figure 3 Western blot analysis. **a**, Skeletal muscle. Muscle extracts from patients 1–6 and 10 and a control were blotted and labelled with antibodies against LAMP-2 (top panel) and β-actin (bottom panel). LAMP-2 is deficient in all muscles examined except the sample from patient 1 that showed a trace amount of protein, which is probably the LAMP-2a isoform. In support of this idea, LAMP-2b is the principal LAMP-2 species in skeletal muscle[6]. Numbers denote patients as in Table 1; C, control. **b**, Heart. Protein was extracted from cardiac muscle of patient 4 and a control, blotted onto a nitrocellulose membrane, and labelled with the antibodies against LAMP-2 (too panel) and β-actin (bottom panel). LAMP-2 is deficient also in cardiac muscle. C, control; 4, patient 4.

Both intronic point mutations and the deletion in an exon–intron junction cause aberrant splicing. Although the point mutation in patient 3 does not disrupt the GT–AG rule of splicing, the mutation site in the fifth nucleotide (G) of an intron is highly conserved (86%) in human genes[7]. We carried out polymerase chain reaction with reverse transcription (RT–PCR) and found that exon 6 was skipped in patient 3 (Fig. 1c).

The mutation present in patients 6–8 disrupted the splice donor-site sequence (GT) in intron 5. We sequenced the RT–PCR product from muscle RNA of patient 6 and found a 6-bp insertion at the junction of exons 5 and 6. The inserted nucleotides had the same sequence as the 5′ end of intron 5. Conceivably, nucleotides 7 and 8 (GT) immediately after the inserted sequence in intron 5 are recognized as an alternative splice-donor site. The inserted sequence encoded an in-frame stop codon (Fig. 1d), which predicted premature termination of the nascent polypeptide (Fig. 2).

The 10-bp deletion in patient 9 ablated four nucleotides in the 3′ end of intron 5, including the splice-acceptor sequence, and six nucleotides in the 5′ end of exon 6. This resulted in a 10-bp deletion at the 5′ end of exon 6, probably because nucleotides 9 and 10 in exon 6, AG, functioned as a new splice-acceptor site (Fig. 1e). In patients 3, 5, 8 and 9, we also identified a polymorphism (A156T) in exon 2, which does not alter the encoded amino acid.

Although patient 1 had a mutation in exon 9b, which should affect only the LAMP-2b isoform, he had the full syndrome with cardiomyopathy, myopathy and mental retardation. This suggests

Table 1 Summary of the *lamp-2* mutations

Patient	Ethnic background	Mutation	Site of mutation	Effect on mRNA	Reference
1	Japanese	2-bp del*	E9b	Frame shift	23
2	Japanese	T440A	E4	Non-sense	24
3	Japanese	g5c	I6	E6 skipping	25, 26
4	Italian	T974AA	E8	Frameshift	27
5	African American	C813G	E8	Non-sense	
6	Japanese	g1a	I5	6-bp insertion	
7	African American	g1a	I5	6-bp insertion §	1
8	Anglo-Saxon	g1a	I5	6-bp insertion §	28
9	Japanese	10-bp deletion†	I5/E6 junction	10-bp deletion ‡	
10	Greek	G14del	E1	Frameshift	29

All patients are male and diagnosed as having DD after muscle biopsy. Exonic mutations (upper case) are shown by the nucleotide position in the LAMP-2b cDNA open reading frame. Intronic mutations (lower case) are described by their position in each intron. E, exon; I, intron; del, deletion; and ins, insertion.
* Deletion of AA at nucleotide positions 1,097 and 1,098.
† Deletions of 4 bp at the 3′ end of I5 and 6 bp at the 5′ end of E6.
‡ Deletion of the sequence corresponding to the 10 nucleotides in the 5′ end of exon 6.
§ Not confirmed in these patients.

that DD is largely due to defects of the LAMP-2b isoform. In support of this idea, LAMP-2b is more abundantly expressed than LAMP-2a in heart, skeletal muscle and brain at the messenger RNA level[6].

The eight distinct *lamp-2* mutations that we identified, except for the exon-skipping mutation in patient 3, abolish both the transmembrane and the cytoplasmic domains of LAMP-2. The exon-skipping mutation in patient 3 also causes extensive structural changes in LAMP-2, not only because it shortens the protein and ablates two glycosylation sites, but also because it abolishes a loop structure by deleting Cys 265, which is thought to form a disulphide bond with Cys 232 (Fig. 2). All the identified mutations are likely to be equally deleterious because there was no apparent phenotypical differences among our patients and no correlation between the genotypes and phenotypes. We did not find any of these mutations in the DNA sequences of 102 control alleles. The A156T was found in 39 alleles. No other polymorphisms were identified in the open reading frame.

To determine the effects of mutations on protein expression, we carried out western blot analysis, immunohistochemistry, or both, on frozen skeletal muscle specimens from all patients except 7 and 8. Western blot analysis showed complete LAMP-2 deficiency in all muscle specimens studied except for the sample from patient 1. The muscle biopsy had a trace amount of the normal size protein, which was most probably the LAMP-2a isoform because patient 1 had a mutation in exon 9b (Fig. 3a). LAMP-2 was also absent in cardiac muscle from patient 4 (Fig. 3b). By immunohistochemistry, the antibody against LAMP-2 failed to detect any structure in DD muscles, whereas the antibody against limp-I, another lysosomal membrane protein, highlighted the intracytoplasmic vacuoles (Fig. 4). The absence of LAMP-2 immunostaining indicates that the protein is probably not retained in endoplasmic reticulum or other membrane-bound structures.

These results strongly suggest that LAMP-2 deficiency is associated with DD. We found mutations in the LAMP-2 gene in 10 of the 11 patients, a clear indication that at least these 10 individuals have primary LAMP-2 deficiency. One patient, who has LAMP-2 deficiency from their immunohistochemistry without an identified mutation in the LAMP-2 coding sequence, may have a mutation in non-coding sequences, including the promoter, intron and other untranslated sequences. Alternatively, the LAMP-2 deficiency in this patient might be secondary. The lack of cardiac symptoms in the mother of this patient favours the second explanation.

The LAMP-2 gene is present on the X chromosome in mouse as in human[8]. LAMP-2-deficient mice show an X-linked disease characterized pathologically by numerous autophagic vacuoles[4]. The presence of vacuolar changes similar to DD, albeit not limited to cardiac and skeletal muscle in LAMP-2-deficient mice, supports our conclusion that DD is caused by primary LAMP-2 deficiency in our 10 patients[4].

The disorders reported[1] as "lysosomal glycogen storage disease with normal acid maltase" are likely to be genetically diverse. Specifically, the rare severely affected infants with unaffected mothers most probably had a different autosomal recessive disease[9,10]. As glycogen is not always increased in this disease, DD is not a glycogen storage disease. We did not include mental retardation as a defining characteristic because it is not always observed and almost impossible to prove in severely affected patients who die in infancy.

X-linked myopathy with excessive autophagia (XMEA) is a similar vacuolar myopathy characterized by intense deposition of the complement C5b-9 membrane attack complex over the muscle fibre surface. On immunostains, the vacuoles in XMEA are also decorated by antibodies to dystrophin and laminin, suggesting a similar pathomechanism to DD[11–13]; however, the vacuoles in an XMEA patient showed strong immunoreactivity both to LAMP-2 and to limp-I, indicating that XMEA is a distinct disease (Fig. 3). In agreement with these findings, sequence analysis of *lamp-2* in another XMEA patient did not show any mutation[6]. Furthermore, the XMEA locus was recently mapped to Xq28 (refs 14, 15), which is distinct from the LAMP-2 locus, Xq24 (ref. 16).

Despite intense investigation, the role of LAMP-2 remains to be fully elucidated. The increased expression of LAMP proteins at the cell surface in metastatic colon cancer and in activated platelets also suggests that it may function in the plasma membrane[17,18]. In DD,

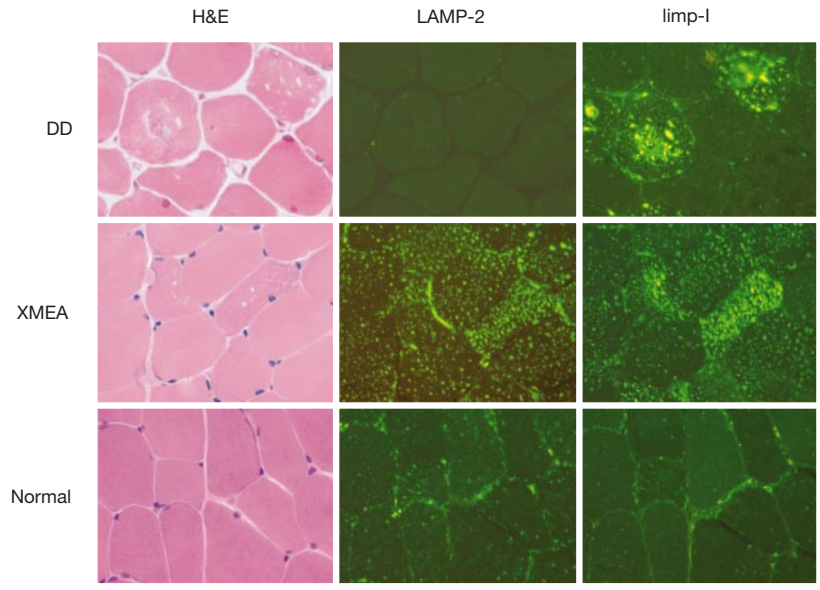

Figure 4 Immunohistochemistry. Serial transverse sections of the biopsied muscle from six DD patients, and samples from a XMEA patient and a normal control, were immunostained with antibodies against LAMP-2 and limp-I. LAMP-2 is absent in all DD muscles (patient 6 shown here), whereas it is present in the XMEA muscle. The antibody against limp-I highlighted vacuoles in both DD and XMEA muscles. LAMP-1 antibody faintly stained both DD and XMEA muscles, suggesting that the expression level of LAMP-1 might be lower in skeletal muscle than LAMP-2 or limp-I (data not shown). The muscle from the patient without a *lamp-2* mutation also showed LAMP-2 deficiency on immunohistochemistry similar to genetically proven DD patients with primary LAMP-2 deficiency (data not shown). The immunohistochemical stains of LAMP-2, limp-I, and LAMP-1 in normal muscle shows numerous tiny particles and accentuates type 1 fibres. H&E, haematoxylin and eosin, original magnification ×150.

the limiting membrane of the endosomal/lysosomal vacuoles are lined by basal lamina and are often continuous with the plasma membrane; this finding might be due to a defect of normal plasma membrane function. Alternatively, the unusual muscle pathology in DD might result from impaired lysosomal protein processing because the cytoplasmic tail of LAMP-2 functions as a receptor for the uptake of certain proteins into lysosome in association with the relative molecular mass (M_r) 73,000 heat shock cognate protein[19].

Our results, together with the recent identification of mutations in the lysosomal proteins cystinosin and sialin that cause nephropathic cystinosis[20] and sialic acid storage diseases[21], respectively, suggest that lysosomal membrane protein defects might not be rare causes of hereditary human diseases. Although all three diseases manifest prominent intracellular vacuoles, the clinical and detailed histological features are distinct. Nephropathic cystinosis typically begins as a renal tubular Fanconi syndrome in the first year of life progressing to renal failure at 9–10 years of age with prominent intracellular crystal formations representing increased cystine stores[22]. By contrast, the sialic acid storage disorders, Salla disease and infantile free sialic acid storage disease are predominantly neurodegenerative conditions with single membrane bound vesicles filled with free sialic acid visible as fibrillogranular material[22]. Sialin and cystinosin are transporter molecules that are thought to export cystine and sialic acid, respectively. By contrast, the function of LAMP-2 is still uncertain. Investigation of this new category of lysosomal diseases will provide important clinical information and yield new insights into the functional roles of the lysosomal membrane and its protein components. □

Methods

Patients

All patients were male and were diagnosed as having DD syndrome on the basis of clinical features and muscle pathology. The four indispensable diagnostic features were cardiomyopathy (confirmed by echocardiogram, electrocardiogram, or both); skeletal muscle weakness on clinical examination; autophagic vacuoles in skeletal muscle; and normal or elevated acid maltase activity. Although not an obligatory feature, mental retardation was seen in eight patients. We excluded one patient with the severe infantile form because inheritance appeared to be autosomal recessive rather than X-linked. Seven of the ten patients were reported previously (Table 1). The patient without *lamp-2* mutation had a total IQ of 92; his mother is healthy, and at age 49 she has a normal electrocardiogram and serum CK level.

Sequence analysis of *lamp-2*

We extracted genomic DNA from blood or skeletal muscle. We amplified each exon and flanking intronic regions with the following primers (the number in the name of the primer indicates exon; F represents forward and R reverse; and sequences are written in 5′ to 3′ orientation): E1F, GAGATTGGCTGTAAGCAAGA; E1R, GACCAGTCTTTCAGGTTGTA; E2F, AGTGGTGGGTAGAGCTTGTT; E2R, CAGTCAACGTGGAATTTCAT; E3F, AAGTGGCATGCCCTACATAA; E3R, GTCAGTGGGAGGGTTATAAA; E4F, GAGAGAAGAAGAAAGCCTAT; E4R, GCCTAGTAGAAGCTATGAAT; E5F, TCTTCCCCTAATATAACCCTTT; E5R, GGAATCATTCTAGTAACACTAC; E6F, AGGCTTTGCTGGCCACTTTT; E6R, CTCCCCCCATGCAACTATTT; E7F, GCTTTTCTTCTGTGGGTATT; E7R, GGTATGATCAAGGTACACTT; E8F, CCTGGCCAACTTTCCCATTT; E8R, GAAAAGCCACCTGTCAACAT; E9aF, GGAAGTGCTGTCATTTTACT; E9aR, CTCAAAATGCTGGGATTGAT; E9bF, CTTTGACTTCGAGACATTCT; E9bR, GTTGACCAGTATTGCATGTT. We directly sequenced the amplified fragments with both forward and reverse primers using BigDye Terminator Cycle Sequencing Kit (PE Biosystems), and then electrophoresed the samples using an ABI PRISM 310 Genetic Analyzer (PE Biosystems). We used PAC clone DJ318C15 sequence (accession number AC002476) as a reference, which includes the entire genomic sequence of LAMP-2 gene in complementary orientation. To determine the effect of the intronic mutations on messenger RNA, we performed RT–PCR in patients 2, 6 and 9. Total RNA was extracted from frozen muscle using Totally RNA Kit (Ambion) and was reverse-transcribed into cDNA with oligo $(dT)_{20}$ primer using the ThermoScript RT–PCR System (Life Technologies). We amplified the cDNA encompassing from exon 1 to exon 9b using primers E1F, TTTTCCCTGGTGTTGCAGCTGTTGTT, and E9bR, GAGTCTGATATCCAGCATAACTTTTT. The amplified fragment was directly sequenced using the PCR primers and relevant internal primers. In patient 10, we only sequenced the RT–PCR product from muscle RNA due to the limited sample availability.

Western blotting

We carried out western blot analyses on skeletal muscle from patients 1–6 and 10, and cardiac muscle from patient 4. Western blot analyses were not performed on patients 7–9 because available muscle samples were inadequate. Frozen tissues were briefly washed with cold PBS, homogenized with triple-detergent lysis buffer (50 mM tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg ml^{-1} phenylmethylsulfonyl fluoride, 1 μg ml^{-1} aprotinin, 1% NP-40, sodium deoxycholate), and spun down. The supernatant was collected and stored at −80 °C until use. We separated 5 μl samples by 7.5% SDS–polyacrylamide gel electrophoresis and electrotransferred the protein onto nitrocellulose membranes at 100 V for 1 h. The membranes were blocked in buffer containing 13.5% fat-free milk, and overlayed with the monoclonal antibody against LAMP-2 (H4B4). The signals were detected using ECL Plus Western Blotting Detection System (Amersham Pharmacia).

Immunohistochemistry

We studied the skeletal muscle from patients 1–3, 5, 6, 9 and the patient in whom we did not find a mutation with immunohistochemical methods. We also analysed the muscles from a normal individual and one patient with XMEA for comparison. Immunohistochemistry was not carried out on patients 4 and 7–9 because available muscle samples were inadequate. Transverse serial sections of 8 μm thickness were cut from each frozen muscle; one section was stained with haematoxylin and eosin and the others were immunostained with the monoclonal antibodies against LAMP-2 (H4B4), limp-I (H5C6) and LAMP-1 (H4A3). The sections for immunostaining were fixed with 4% formaldehyde in 0.1 M $CaCl_2$ (pH 7.2), followed by dehydration in ethanol series, and blocked with 10% fetal bovine serum in PBS. The monoclonal antibodies were applied with 1:50 dilution. The sections were subsequently incubated with biotinylated anti-mouse IgG (Amersham Pharmacia) with 1:100 dilution and fluorescein-conjugated streptavidin (Amersham Pharmacia) with 1:250 dilution. Signals were detected under Optiphot-2 fluorescent microscope (Nikon). All the antibodies used in this study were developed by J. T. August and J. E. K. Hildreth and were obtained from Developmental Studies Hybridoma Bank (University of Iowa).

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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

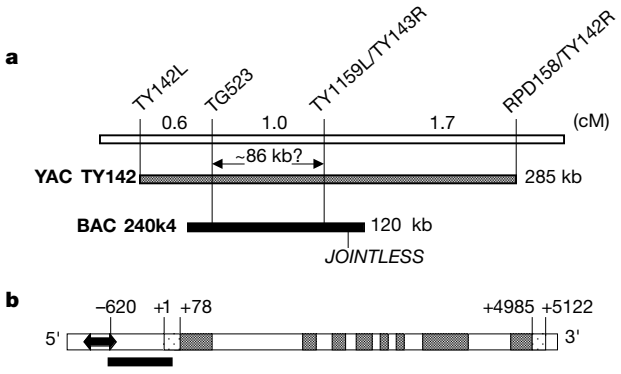

Figure 1 The tomato *JOINTLESS* gene. **a**, Genetic map of the locus on chromosome 11. The TY142L and TY142R are ends of TY142. *Jointless* co-segregates with two other YAC ends TY159L and TY143R. The BAC clone 240k4 contains the flanking genetic marker TG523 and YAC end TY159L. **b**, Diagram of the *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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