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Abstract

Background: Ubiquitin-dependent protein degradation is a critical step in key cell cycle events, such as metaphase-anaphase transition and mitotic exit. The anaphase promoting complex/cyclosome (APC/C) plays a pivotal role in these transitions by recognizing and marking regulatory proteins for proteasomal degradation. Its overall structure and function has been elucidated mostly in yeasts and mammalian cell lines. The APC/C is, however, a multisubunit assembly with at least 13 subunits and their function and interaction within the complex is still relatively uncharacterized, particularly in metazoan systems. Here, lemming (lmg) mutants were used to study the APC/C subunit, Apc11, and its interaction partners in Drosophila melanogaster.

Results: The lmg gene was initially identified through a pharate adult lethal P element insertion mutation expressing developmental abnormalities and widespread apoptosis in larval imaginal discs and pupal abdominal histoblasts. Larval neuroblasts were observed to arrest mitosis in a metaphase-like state with highly condensed, scattered chromosomes and frequent polyploidy. These neuroblasts contain high levels of both cyclin A and cyclin B. The lmg gene was cloned by virtue of the lmg03424 P element insertion which is located in the 5′ untranslated region. The lemming locus is transcribed to give a 2.0 kb mRNA that contains two ORFs, lmgA and lmgB. The lmgA ORF codes for a putative protein with more than 80% sequence homology to the APC11 subunit of the human APC/C. The 85 amino acid protein also contains a RING-finger motif characteristic of known APC11 subunits. The lmgA ORF alone was sufficient to rescue the lethal and mitotic phenotypes of the lmg138 null allele and to complement the temperature sensitive lethal phenotype of the APC11-myc9 budding yeast mutant. The LmgA protein interacts with Mr/Apc2, and they together form a binding site for Vihar, the E2-C type ubiquitin conjugating enzyme. Despite being conserved among Drosophila species, the LmgB protein is not required for viability or fertility.

Conclusions: Our work provides insight into the subunit structure of the Drosophila APC/C with implications for its function. Based on the presented data, we suggest that the Lmg/Apc11 subunit recruits the E2-C type ubiquitin conjugating enzyme, Vihar, to the APC/C together with Mr/Apc2 by forming a ternary complex.

Background

Chromosome separation at anaphase onset and exit from mitosis are regulated by ubiquitylation and subsequent degradation of key regulatory proteins, the securins and mitotic cyclins [1]. The ubiquitylation of these proteins is catalyzed by a cascade of E1, E2 and E3 enzymes, the crucial factor being the cell cycle regulated E3 ubiquitin protein ligase, the anaphase-promoting complex or cyclosome (APC/C) that provides the platform for the ubiquitylation reaction and determines substrate specificity.

The APC/C contains at least 13 different subunits in the budding yeast, Saccharomyces cerevisiae, and most of these subunits appear to be conserved in all eukaryotes with the exception of Apc9 to which no homologs have been identified in multicellular eukaryotes. In contrast,
the Apc7 and Apc16 homologs have been identified in multicellular, but not in unicellular, eukaryotes [2,3].

An architectural analysis of the budding yeast APC/C revealed two subcomplexes that are held together by the largest subunit, Apc1 [4]. Apc1, together with subunits Apc4 and Apc5 serves as a scaffold for the whole complex. One of the subcomplexes connects through the Apc4 and Apc5 subunits and contains three subunits (Cdc16, Cdc23 and Cdc27), with tandem arrays of multiple tetra-tricopeptide repeats (TPR). Since the TPR motifs are generally involved in protein-protein interactions, they are thought to contribute to substrate binding. The other APC/C subcomplex contains Apc2, Apc10/Doc1 and Apc11 subunits, and connects to Apc1 through Apc2 [4]. It has been proposed that in yeast and in human cells, either the RING finger Apc11 subunit alone [5,6], or together with the cullin homolog Apc2 defines the minimal ubiquitin ligase activity of the APC/C, depending on the type of ubiquitin conjugating enzyme (E2) used in these reactions [7]. The gene encoding Apc11 has been cloned from the nematode, Caenorhabditis elegans. RNAi analysis in this species has shown that in the absence of Apc11, zygotes arrested before the onset of cleavage. The absence of polar bodies in these zygotes indicates arrest during the first meiotic division of the oocyte [8]. In Arabidopsis thaliana, Apc11 interacts with APC2 and form a heterodimer complex [9]. In HeLa cells hydrogen peroxide induces zinc release from APC11, and impairs the interaction between APC11 and the E2 enzyme Ubc4 and therefore inhibits the ubiquitin ligase activity of APC11 [10]. The functions of other subunits remain obscure, though some of them, like Apc13 and Cdc26 were implicated in stabilizing the complex [11].

To understand the role of APC/C subunits in Drosophila, we previously undertook detailed genetic and molecular analyses of the genes coding for several subunits. We have shown that the Mákos/Cdc27, Cdc16 and Cdc23 TPR subunits and the Apc10/Doc1 subunit were essential and required to mediate progression through metaphase. The fourth TPR subunit, Apc7 is a nonessential component of the Drosophila APC/C with unknown function [12-14]. In this paper we present mutational and molecular analysis of the Drosophila lemming (lmg) gene and show that it has an unusual dicistronic mRNA. The upstream open reading frame (ORF), lmgA is an essential gene that encodes a functional homologue of the S. cerevisiae small APC/C subunit, Apc11. The downstream ORF, lmgB is dispensable without causing any detectable phenotype. Phenotypic analysis of hypomorphic and null lmg alleles revealed prometa- phase cell cycle arrest and apoptosis, consistent with loss of APC/C function. Interacting partners of Lmg/Apc11 were also investigated by yeast two-hybrid analysis and their possible function will be discussed.

Results
Disruption of lemming results in pupal lethality and widespread apoptosis
The l(2)03424 mutant stock was originally identified during a screen of the Berkeley Drosophila Genome Project (BDGP) collection of P element insertion lines for pha- rate adult lethal mutants with defective abdomens. l(2) 03424 homozygotes die at pharate adult stage P15(i) (Table 1) [15] and additionally have reduced eyes and wings, as well as bristle defects. Acridine orange and TUNEL staining of imaginal discs showed that a likely cause of these defects was apoptosis of imaginal cells (data not shown). The gene containing the P element insertion was re-named lemming (lmg) and its first mutant allele was designated lmg<sup>03424</sup>. The P element was confirmed as the cause of the lmg<sup>03424</sup> mutation, as precise excisions reverted to a wild type phenotype. A stronger allele, lmg<sup>0223</sup> was established by imprecise excision of the P element by crossing to a Δ2-3 transposase-source strain. Most of the lmg<sup>0223</sup> homozygotes die as P5 (i) stage pupae (Table 1). Orcein and acridine orange staining of larval brains and imaginal discs revealed widespread apoptosis (Figure 1). In addition, a non-comple- menting P element insertion allele, lmg<sup>EX11157</sup> identified by the BDGP, showed P4(ii) lethality, and the imprecise excision of its P element yielded the strongest lemming allele, lmg<sup>138</sup>, with a lethal phase in P4(i) (Table 1). The lmg<sup>138</sup> mutation proved to represent a small deletion removing almost the entire coding region of the gene (Figure 2A); therefore it is considered a null allele of lemming. Dissected tissues showed that the optic lobes and imaginal discs of lmg<sup>138</sup> homozygotes were substantially reduced in size compared to wild type (Figure 1F).

lemming mutants show metaphase-like delay with overcondensed chromosomes
The apoptotic phenotype of lmg mutants is restricted to mitotically active cells suggesting that it may be caused by mitotic defects. We examined orcein-stained brain squash preparations for mitotic abnormalities from lmg mutant third instar larvae. Examples of mitotic figures in lmg neuroblasts are shown in Figure 3. Chromosomes appeared normal during prophase, but overcondensation was readily apparent at prometaphase and/or metaphase (Figure 3D-K). The proportion of cells in mitosis was much higher in the mutant preparations compared to wild type, most of the dividing cells being in a prometa- phase- or metaphase-like stage. Cells in anaphase were rare and most of them appeared abnormal with highly condensed (Figure 3N and 3O), lagging chromosomes (Figure 3L and 3M). Polyploid cells with overcondensed chromosomes were frequently observed (~ in 30% of cells in mitosis; Figure 3G, K and 3O).
Larval brains of lmg03424, lmgEY11317 and lmgJ023 had a high mitotic index (4.1 for lmg03424, 5.8 for lmgEY11317 and 4.4 for lmgJ023, n = 135, 170 and 167 respectively), compared to w1118 (1.6), (Table 1). The high mitotic index and the extent of chromosome overcondensation are indicative of metaphase arrest. This is further supported by the high metaphase to anaphase ratios (4.5 for lmg03424, 6.2 for lmgEY11317 and 6.3 for lmgJ023). It should be mentioned that though anaphase still occurs at a lower rate in lmg mutants, true metaphase figures with chromosomes fully aligned to the metaphase plate were never observed, in spite of the fact, that such an alignment could be identified in wild type preparations in about 4-6% of mitotic cells. Instead, the cells persisted in a prometaphase- or metaphase-like state with scattered, highly condensed chromosomes.

**lemming** mutants accumulate cyclin A and B

Mitotic cyclins are one of the prime APC/C substrates. The APC/C targets the mitotic cyclins for degradation in late mitosis, thereby permitting mitotic exit [16]. We have shown previously, that Apc3/Cdc27, Apc6/Cdc16 and Apc10 mutants show elevated levels of cyclin A and cyclin B [12-14]. Therefore we tested if this function was affected in lmg mutants by monitoring cyclin levels in wild type and lmg mutant neuroblasts in immunostaining experiments with polyclonal antibodies against cyclin A and cyclin B. In wild type, a high level of cyclin A could be detected in prophase and early prometaphase cells (Figure 4A and 4D), identifiable on the basis of weaker and somewhat diffuse DNA signal, but it disappeared during metaphase (Figure 4B and 4E). Cyclin B was highly visible in prometaphase (Figure 5A and 5D), it started to disappear in metaphase (Figure 5B and 5E), and was undetectable in anaphase cells. Only 14% and 17% of cells with bright DNA staining retained cyclin A (n = 29) and cyclin B (n = 24) signals respectively. Both antisera gave strong staining in lmg mutant preparations as well. However, in contrast to wild type, more than 80% of lmgJ023 neuroblasts that showed intense DNA staining around the middle of the spindle, indicating arrest in a metaphase-like state, also showed visible accumulation of both cyclin A (n = 17; Figure 4C and 4F) and cyclin B (n = 20; Figure 5C and 5F), most abundantly around the mitotic spindle. This phenotype was similar in all lmg mutants, and indicates that the Lmg protein is required for cyclin A and cyclin B degradation.

**The lemming locus is dicistronic and its upstream ORF, lmgA, codes for the Apc11 subunit of the Drosophila APC/C**

The P element insertions of lmg03424 and lmgEY11317 mutants were both mapped to the left arm of chromosome 2, to the cytogenetic location 29D4-5. Remobilization of these insertions resulted in frequent reversion of the mutant phenotype to wild type, suggesting that these

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**Table 1 Quantitative analysis of lethal and mitotic phenotypes of lmg mutants**

| Genotype | Lethal phase | Preparations per fields | Mitotic index | M:A ratio | Polyploid % | Apoptotic index |
|----------|--------------|-------------------------|---------------|-----------|-------------|----------------|
| **A**    |              |                         |               |           |             |                |
| w1118    | Viable      | 7/183                   | 1.6 ± 0.2     | 2.9 ± 0.3 | 0           | 0.5 ± 0.1      |
| lmg03424 | Pharate adult P15(i) | 6/135                      | 4.1 ± 0.8     | 4.5 ± 0.7 | 196 ± 56    | 1.4 ± 0.3      |
| lmgJ023  | Pupal stage P5(i) | 7/167                      | 4.4 ± 0.9     | 6.3 ± 0.1 | 291 ± 74    | 1.2 ± 0.2      |
| lmgEY11317 | Preparupal stage P4(ii) | 6/170                      | 5.8 ± 0.7     | 6.2 ± 0.4 | 266 ± 3.1   | 1.5 ± 0.4      |
| lmgJ023  | Preparupal stage P4(ii) | 10/194                     | 5.9 ± 0.5     |        | 507 ± 7.9   | 1.8 ± 0.5      |
| lmgJ023 lmgA- pUAST/dgaGAL4 | Viable | 6/180                     | 1.5 ± 0.2     | 3 ± 0.6   | 0           | 0.5 ± 0.1      |
| lmgJ023 lmgA + B- pUAST/dgaGAL4 | Viable | 4/100                     | 1.4 ± 0.2     | 3.1 ± 0.3 | 0           | 0.6 ± 0.2      |
| lmgJ023 lmgB- pUAST/dgaGAL4 | Preparupal stage P4(ii) | 5/100                     | 4.9 ± 0.9     |        | 437 ± 6.2   | 1.7 ± 0.2      |
| **B**    |              |                         |               |           |             |                |
| mr2      | Female sterile | 6/174                     | 1.4 ± 0.1     | 3.3 ± 0.4 | 0           | 1.2 ± 0.3      |
| lmg03424, mr2 | Prepupal stage P4(ii) | 6/106                     | 4.5 ± 0.7     | 4.4 ± 0.3 | 318 ± 3.9   | 1.3 ± 0.4      |
| **C**    |              |                         |               |           |             |                |
| viharS11001 | Pharate adult P15(i) | 7/196                     | 2.1 ± 0.3     | 3.7 ± 0.6 | 4.2 ± 0.4   | 0.9 ± 0.3      |
| lmg03424, viharS11001 | Bubble prepupa | 6/116                     | 6.1 ± 0.6     | 39 ± 0.7  | 443 ± 8.4   | 1.8 ± 0.3      |
| mtf, viharS11001 | Early L3 larvae | n.d.                      | n.d.          | n.d.      | n.d.        | n.d.           |
| mtf, viharS11001 | Early L3 larvae | n.d.                      | n.d.          | n.d.      | n.d.        | n.d.           |

The number of preparations and the total number of optical fields analyzed are given. Mitotic index is given as the average number of mitotic cells in an optical field (± sd). Polyploid% represents the percentage of mitotic cells (± sd) with higher than two sets of chromosomes. Apoptotic index is given as the mean number of apoptotic cells in a microscope field (± sd).
insertions were directly responsible for disrupting gene function. Genomic DNA flanking the \textit{lmg}\textsubscript{03424} insertion was recovered by plasmid rescue and used to probe larval cDNA libraries. Several overlapping clones were identified and their nucleotide sequences determined. BLAST searches of the Berkeley \textit{Drosophila} Genome Project (BDGP) collection of ESTs revealed a number of embryo-nic cDNA clones which shared nucleotide sequence with the putative \textit{lmg} cDNAs. One of these (LD20119) was completely sequenced and comparison with genomic sequence showed that the \textit{lmg} locus is intronless. The 2.0 kb transcript contains two ORFs separated by 92 bases (Figure 2A). Sequence data of 423 cDNA clones for this locus are reported in FlyBase. They identify two cDNA classes based on size, a 2.0 and a 2.9 kb, both of which appear to be dicistronic. The 5'-ends of 230 cDNA clones identifies the transcription start site at 192 (± 4) bps upstream of the ORF1 start codon [17]. To better characterize the transcription profile of the \textit{lemming} locus, RACE analyses were performed, using total RNA isolated from embryos and L3 larvae. Regardless of RNA source, the 5'-end of the transcript (using the ORF1 inner primer) could be placed at 180 (± 15) bases upstream of the ORF1 start. However, 5'-RACE analysis with the 5' ORF2 inner primer on embryonic RNA revealed two different 5'-ends: one extending 107 bases upstream of the ORF2 translation start site and the other one matching the 5'-end of ORF1 (Figure 2A). Similar 5'-RACE analysis (with the 5' ORF2 outer primer) on L3 larval RNA resulted in only one mRNA 5'-end that matched the 5'-end of embryonic mRNA upstream of ORF1, thus representing dicistronic mRNA. The 3' RACE experiments using ORF1 primers yielded an mRNA end located in the intercistronic region 56 bases downstream of the stop codon of ORF1. The determination of the 3'-end using ORF2 primers was ambiguous by 3' RACE, but sequencing the 3'-end of the cDNA clone identified in the yeast two hybrid experiments (see later) revealed the 3'-end at 1231 bases downstream of

\textbf{Figure 1} Loss of \textit{lmg} leads to elevated apoptosis in larval brain and imaginal discs. Orcein-stained preparations of wild-type (A) and \textit{lmg}\textsuperscript{138} (D) larval brain squashes. \textit{lmg} mutants show small, rounded cells with uneven nuclear staining. Acridine Orange staining also highlights the high incidence of dying cells in \textit{lmg}\textsuperscript{1033} mutant wing imaginal disc (B) and \textit{lmg}\textsuperscript{138} larval brain (F) relative to wild-type tissues (B and C). Green dots indicate apoptosis. The \textit{lmg}\textsuperscript{1033} null mutant (F) has reduced brain and optic lobes compared to wild-type (C).
Figure 2 Schematic representation of the \textit{lmg} locus. The molecular map of the \textit{lmg} locus represents the ~2.0 kb cDNA sequence available in FlyBase (A). It indicates the two ORFs (rectangles), transcription start sites (TSS1 and 2), P element insertion sites (triangle), the extension of deletion in the \textit{lmg}^{J023} and \textit{lmg}^{138} alleles and the 3'-end of the monocistronic transcript identified by RACE. It also shows the relative location of RACE primers used in this work. The primer pairs used for developmental RT-PCR experiments and their relative locations are also displayed (B).

Figure 3 Mitotic abnormalities of \textit{lmg} alleles in orcein stained larval neuroblasts. Wild-type mitotic cells in prometaphase (A), metaphase (B) and anaphase (C). Neuroblast cells from different \textit{lmg} mutants show prometaphase- or metaphase-like arrest with overcondensed chromosomes and polyplody (D-K). Chromosome overcondensation could also be observed in anaphase figures (N and O), together with scattered chromosome segregation (L and M). Many cells appear polyplody (G, K and O), and they invariably have overcondensed chromosomes.
Mitotic cyclin A is not degraded in *lmg* mutants. Images show cyclin A (red in A, B, C, monochromatic in D, E, F), and tubulin (green in A, B, C, monochromatic in G, H, I) localizations as well as DNA staining (blue in A, B, C, monochromatic in J, K, L) in mitotic cells. In wild-type cells (*n* = 29) the cyclin A staining is visible in prophase or prometaphase (A and D), but undetectable in metaphase cells (B and E). In metaphase-like *lmg*<sup>J023</sup> cells (*n* = 17) cyclin A staining is intense (C and F).
Figure 5 CyclinB is not degraded in Img mutants. Colours are the same as in Figure 4. The level of Cyclin B is high in wild-type (n = 24) prophase and prometaphase cells (A and D) and it starts to disappear at or after the onset of metaphase (B and E). Cyclin B staining is quite pronounced in most of the Img<sup>1023</sup> (C and F) cells (n = 20) arrested in metaphase.
the stop codon closing ORF2. Based on this analysis, we concluded that the lmg locus gives rise to a bona fide dicistronic message and designated ORF1 and ORF2 as lmgA and lmgB respectively. The presence of monocistronic mRNAs could be explained by processing of the dicistronic messages, although it is still possible that original monocistronic mRNAs may be made.

The lmgA ORF encodes a small, putative polypeptide of 85 amino acids (~ 10 kDa), that contains a RING-finger motif characteristic of known APC11 subunits, and shows more than 80% sequence similarity with the APC11 subunit of the human APC/C. The lmg ORF (corresponding to CG34441 in FlyBase) codes for a putative polypeptide of 365 amino acids, with no apparent functional domains. In a more detailed bioinformatic analysis of the lemming locus, the evolutionary conservation of lmgA, the 92 bp intercistronic sequence (ICS), lmgB and the 3’ untranslated region (3’-UTR) of 12 Drosophilidae species were determined and compared. As Table 2 illustrates, the topology of the locus is maintained in these species, but the conservation of the different regions shows considerable variations. The amino acid sequence of LmgA shows the highest conservation (96% at minimum), while LmgB shows only 66% identity between the most widely diverged species. Though the ICS region varies in length (92-165 bps), together with the 3’-UTR, they still demonstrate a significant level of conservation among the Drosophila species analysed. Although the LmgB protein sequence is conserved in Drosophilidae, homologous sequences could not be found in other species.

The developmental transcription pattern of lmgA and lmgB is very similar

Dicistronic genes are often products of a duplication event and related functionally. However, neither the nucleotide, nor the amino acid sequence of ORF1 and ORF2 showed any similarity. Nonetheless, the existence of mono- and dicistronic mRNAs raised the possibility of coordinated expression. To investigate this, total RNA was isolated from isogenized w^{1118} embryos (0-12 hours), L1, L2, early L3, late L3 larvae, early pupae, late pupae, males and females. These samples were then used to analyze the expression pattern of monocistronic and dicistronic messages by semi-quantitative RT-PCR, using lmgA, lmgB and lmgAB (spanning the intercistronic region) specific primer pairs (Figure 2B and Materials and Methods). As Figure 6A shows, the expression patterns of these transcripts are very similar. In each case, lowest expression occurs in embryos, followed by a significant increase in first instar larvae. Low expression could be detected in second and early third instar larvae, and then it intensifies in late third instar larvae and early pupae. Late pupae show the highest expression level for these transcripts that then stays steady in male and female adults. This result implies that the dicistronic message is the main product of lmg transcription. If monocistronic lmgA or lmgB messages are present, their expression profile does not differ significantly from that of the dicistronic message.

The lmgB ORF is not efficiently translated

Translation usually starts at the AUG codon of the upstream cistron, and it is suggested that translation of the second cistron of a dicistronic mRNA is initiated at an internal ribosome entry site (IRES) or by some kind of reinitiating processes [18]. Our in silico sequence analysis did not identify predicted IRES sites in the intercistronic region. In order to test the translation of the two ORFs, the dicistronic cDNA was cloned into a Gateway expression vector, in which a hemagglutinin (HA) tag was inserted in frame to the 5’-end of lmgA, and a Flag

| Species          | LmgA (aa%) | 92 ICS(bp) % | LmgB (aa%) | 3’-UTR (bp)% |
|------------------|------------|--------------|------------|--------------|
| D. simulans      | 100        | (92) 97      | 145 aa, 90 | 92           |
|                  |            |              | 143 aa, 99 |              |
| D. sechellia     | 100        | (90) 100     | 98         | 96           |
| D. yakuba        | 100        | (83) 88      | 98         | 91           |
| D. erecta        | 100        | (83) 86      | 97         | 91           |
| D. ananassae     | 98         | (96) 53      | 84         | 67           |
| D. pseudoobscura | 98         | (111) 41     | 77         | 58           |
| D. persimilis    | 98         | (111) 41     | 76         | 59           |
| D. willistoni    | 97         | (157) 28     | 69         | 47           |
| D. mojavensis    | 97         | (165) 33     | 67         | 47           |
| D. virilis       | 96         | (162) 31     | 66         | 47           |
| D. grimshawi     | 97         | (150) 36     | 67         | 46           |

aa% and bp% represent amino acid and nucleic acid sequence similarities in different regions of the lemming locus from 11 species compared to D. melanogaster sequences.

ICS: intercistronic sequence; 3’-UTR: 3’-untranslated region

The developmental transcription pattern of lmgA and lmgB is very similar

Dicistronic genes are often products of a duplication event and related functionally. However, neither the nucleotide, nor the amino acid sequence of ORF1 and ORF2 showed any similarity. Nonetheless, the existence of mono- and dicistronic mRNAs raised the possibility of coordinated expression. To investigate this, total RNA was isolated from isogenized w^{1118} embryos (0-12 hours), L1, L2, early L3, late L3 larvae, early pupae, late pupae, males and females. These samples were then used to analyze the expression pattern of monocistronic and dicistronic messages by semi-quantitative RT-PCR, using lmgA, lmgB and lmgAB (spanning the intercistronic region) specific primer pairs (Figure 2B and Materials and Methods). As Figure 6A shows, the expression patterns of these transcripts are very similar. In each case, lowest expression occurs in embryos, followed by a significant increase in first instar larvae. Low expression could be detected in second and early third instar larvae, and then it intensifies in late third instar larvae and early pupae. Late pupae show the highest expression level for these transcripts that then stays steady in male and female adults. This result implies that the dicistronic message is the main product of lmg transcription. If monocistronic lmgA or lmgB messages are present, their expression profile does not differ significantly from that of the dicistronic message.

The lmgB ORF is not efficiently translated

Translation usually starts at the AUG codon of the upstream cistron, and it is suggested that translation of the second cistron of a dicistronic mRNA is initiated at an internal ribosome entry site (IRES) or by some kind of reinitiating processes [18]. Our in silico sequence analysis did not identify predicted IRES sites in the intercistronic region. In order to test the translation of the two ORFs, the dicistronic cDNA was cloned into a Gateway expression vector, in which a hemagglutinin (HA) tag was inserted in frame to the 5’-end of lmgA, and a Flag

| Species          | LmgA (aa%) | 92 ICS(bp) % | LmgB (aa%) | 3’-UTR (bp)% |
|------------------|------------|--------------|------------|--------------|
| D. simulans      | 100        | (92) 97      | 145 aa, 90 | 92           |
|                  |            |              | 143 aa, 99 |              |
| D. sechellia     | 100        | (90) 100     | 98         | 96           |
| D. yakuba        | 100        | (83) 88      | 98         | 91           |
| D. erecta        | 100        | (83) 86      | 97         | 91           |
| D. ananassae     | 98         | (96) 53      | 84         | 67           |
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| D. willistoni    | 97         | (157) 28     | 69         | 47           |
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| D. grimshawi     | 97         | (150) 36     | 67         | 46           |

aa% and bp% represent amino acid and nucleic acid sequence similarities in different regions of the lemming locus from 11 species compared to D. melanogaster sequences.

ICS: intercistronic sequence; 3’-UTR: 3’-untranslated region
tag was ligated to the 3'-end of lmgB. Schneider 2 (S2) cells were transfected with this construct and, after propagation, total protein extract was prepared and analyzed by Western blots using anti-Flag and anti-HA monoclonal antibodies. As Figure 6B illustrates, while HA-tagged LmgA was produced, no LmgB-Flag protein could be detected. Thus, while the expression pattern of the dicistronic mRNA could be monitored throughout development and it was expressed in S2 cells, the translation of the downstream lmgB ORF could not be detected. However, it cannot be excluded that LmgB might be produced below detection sensitivity. It is also possible that by unfortunate coincidence, the LmgB-Flag protein migrates with a lower electrophoretic mobility than expected, resulting in comigration with the band recognized by anti-Flag nonspecifically (Figure 6B).

Expression of lmgA alone rescues the lethal, morphological and mitotic phenotypes of the lmg138 mutant

In order to determine if one or both ORFs were required for essential lemming functions, transgenic lines were established in a lmg138 mutant background expressing either dicistronic (lmgAB) cDNA, or the lmgA or lmgB ORFs alone under control of the yeast transcription factor Gal4 [19]. The ability of these transgenes to rescue the lethal, morphological and mitotic phenotypes of lmg138 was investigated. As Table 1 demonstrates, ubiquitous expression of the lmgAB or lmgA transgenes by the da-Gal4 driver was sufficient to rescue fully the lethal and mitotic phenotypes of lmg138, and to restore the normal development of imaginal discs and larval brain. In contrast, the ubiquitous expression of lmgB rescued neither the lethal nor the mitotic mutant phenotype in several independent transgenic lines, despite the fact that its expression at mRNA level could easily be detected by RT-PCR. These data demonstrate that the developmental and mitotic defects observed in lmg mutants reflect essential functions of lmgA. On the other hand, lmgB appears to be dispensable without any detectable effect.

Heterologous expression of LmgA complements a S. cerevisiae apc11 mutant

Since the predicted LmgA protein showed a very high degree of similarity to yeast and human Apc11 proteins, we wanted to test if LmgA was able to function as an Apc11 substitute in budding yeast cells defective in their endogenous APC11 function. For this, we introduced a lmgA expression construct under the control of the constitutively active alcohol dehydrogenase promoter into APC11-myc9 cells and tested for complementation of their temperature sensitive proliferation defect [11]. As Figure 7A and 7B show, APC11-myc9 cells grow well at 30°C but are unable to do so at 37°C, though the W303 control cells are quite capable of growing at the higher, restrictive temperature (Figure 7B). The ability of APC11-myc9 cells to grow at 37°C was restored

Figure 6 Expression pattern of different lmg gene products. Semi-quantitative RT-PCR was used to monitor the lmg-specific transcripts in total RNA samples from animals at different developmental stages (A). Western blots of total protein extracts from S2 cells transfected with the dicistronic lmgAB construct treated with anti-HA or anti-FLAG monoclonal antibodies (B). Even after overloading the anti-FLAG track, no FLAG-tagged LmgB could be detected. The arrow indicates the predicted molecular weight of LmgB. Bands labeled with asterisks are polypeptides nonspecifically recognized by anti-FLAG and anti-HA antibodies. Ctrl: Total protein extract from non-transfected cells treated with anti-FLAG or anti-HA antibodies.
following transformation with the pRS426-lmgA plasmid, which expresses LmgA constitutively. In parallel experiments, the APC11-myc9 cells failed to grow at the restrictive temperature when transformed with the empty vector (pRS426), or with the pRS426-mks plasmid expressing the Apc3 subunit of the Drosophila APC/C (Figure 7B). This result proves that the LmgA protein functionally complements the APC11 subunit in yeast and it suggests that the function of the Apc11 homologues is evolutionarily conserved.

LmgA interacts with Apc2, and together, they bind Vihar. As it is known that the yeast and human APC2 protein interacts with APC11 and together they form the catalytic subcomplex of the APC/C [4], we examined if the Drosophila proteins interacted similarly. In a yeast two-hybrid (Y2H) assay, apc2-pBTM116 served as bait to screen a Drosophila embryo cDNA library cloned into the pACT2 vector (Clontech Laboratories, Inc., USA). Originally, this experiment served to identify Apc2 interacting proteins. A strong interaction was found with a clone that was purified and characterized. Sequence analysis showed that it contains two tandem ORFs in different frames matching the dicistronic mRNA of the lemming gene. To identify the region of the lmg cDNA required for apc2-pBTM116 interaction, the two segments corresponding to lmgA and lmgB were cloned into pGAD424 and examined in two-hybrid
assays. Only LmgA showed strong interaction with Apc2, even if the fusions to the activation and binding domains were reversed (Figure 8A). A yeast two hybrid screen was also performed to find interacting partners for the hypothetical protein coded by lmgB. Using lmgB-pBTM116 as bait, no interacting clone could be identified in the cDNA library mentioned above.

It has been shown that Ubc4 and E2-C type E2s bind in vitro either to the Apc2 or Apc11 subunit of the catalytic subcomplex [7]. However, genetic analysis of E2 mutants in yeast and Drosophila suggests that, in vivo, the E2-C type enzymes are required for physiological APC/C function [20,21]. There is only one E2-C enzyme in Drosophila, encoded by the vihar gene [21]. Since the binding partner of Vihar and the nature of its interaction were not known, we used yeast two-hybrid assays to test for physical interactions between Vihar and either Apc2/Mr, or Apc11/LmgA. Though fusions of vihar, Apc2/mr and Apc11/LmgA coding sequences were made to all possible activation and binding domain combinations, no interactions could be detected. A possible explanation of this result is that the Apc2/Mr and Apc11/LmgAsubunits jointly create a binding site for Vihar, so the three proteins could form a ternary complex. To assess this possibility, we used yeast three-hybrid assays in which interactions between lmgA-pBTM116, vihar-pGAD424 and the methionine-regulated apc2-pRS416 (see Materials and Methods) were analyzed. In this setting, interaction among these proteins was clearly observed (Figure 8B and 8C), indicating that both Apc2/Mr and Apc11/LmgA are required for Vihar binding.

Discussion
The APC/C belongs to the cullin-RING family of multi-subunit ubiquitin ligases. Previous studies of the budding yeast and human APC/C indicated that the cullin-related Apc2 and the RING-finger-containing Apc11 subunits together form the minimal ubiquitin ligase module [4,7]. We show in this paper that, in Drosophila melanogaster, the Apc11 subunit is encoded by the dicistronic lemmin locus. The upstream ORF, lmgA,
encodes a putative protein containing a RING-finger motif characteristic of known APC11 subunits and shows more than 80% sequence similarity with the APC11 subunit of the human APC/C. Since the Apc11 subunit is proposed to play a role in the catalytic center of the APC/C, mutations in lmg are expected to lead to loss of APC function, and therefore to aberrant cell cycle progression. The lmg mitotic phenotype presented in this paper is consistent with the Lmg protein being a subunit of the APC. The mitotic defects we observe in lmg larval neuroblasts, including metaphase-like arrest, chromosome overcondensation and polyploidy, in addition to widespread apoptosis of mitotically-active cells, are very similar to those reported for loss of other subunits of the Drosophila APC/C [12-14]. A role of LmgA in the APC/C is further supported by the elevated levels of cyclin A and B observed in lmg neuroblasts. Another line of supporting evidence comes from the synergistic genetic interaction between lmgA and mr/Apc2 and lmgA and vihar (Table 1B and 1C) and from the physical interactions among these proteins, since it is known that, in yeasts and vertebrates, these proteins form the catalytic module of the APC/C. These data, together with its ability to complement the mutant phenotype of yeast Apc11-deficient cells support the designation of lmgA as a true Apc11 orthologue.

The APC/C requires special E2 enzymes for activity and has been demonstrated to function with Ubc4/5 and E2-C type E2 enzymes in vitro [22,23]. Whereas in yeast and human cells the E2 enzymes bind to either Apc2 or Apc11, our data suggest that in Drosophila, both of these subunits are required for effective E2 binding. This could represent an architectural variation in the catalytic subcomplex of different APC/C ligases.

The dicistronic nature of the lmg locus is a notable but puzzling fact. Whereas the upstream lmgA ORF encodes the Apc11 subunit of the Drosophila APC/C, the existence and function of the predicted downstream lmgB ORF product remains unknown. We could not find any sequence or functional relationship between lmgA and lmgB, though such relationships are characteristic of many dicistronic genes [24]. Genomes of other species from Drosophilidae (especially in the melanogaster group) contain both these ORFs and the intercistronic sequence in a similar arrangement (data not shown). Moreover, the high evolutionary conservation of LmgA and LmgB and significant conservation of both ICS and 3’-UTR suggest functional relevance. However, we found that the putative LmgB is dispensable for the organism and lacks known protein motifs. In addition to this, no apparent LmgB interaction partners could be found in yeast two hybrid screen and LmgB could not be efficiently translated from the dicistronic mRNA in S2 cells. lmgA contains three in-frame AUG codons in addition to its initiating AUG codon. It has been shown for two Drosophila dicistronic transcripts, of the stoned and snapin loci, that such in-frame AUG codons effectively attenuate the translation of the second ORF [25]. However, the rationale for the dicistronic arrangement of the lmgA and lmgB cistrons and the function of the lmgB ORF remains obscure.

The mechanism by which loss of APC/C function leads to apoptosis is unknown but it may be significant that lmg mutant cells entered apoptosis directly, and rapidly, from arrested cells, without a return to the interphase state (data not shown). There is accumulating evidence that mitosis and apoptosis share components [26]. It has been suggested that apoptosis is a default pathway and proteins such as survivin are required to counteract this pathway during mitosis [27,28]. Cells treated with drugs which alter microtubule dynamics, such as paclitaxel (Taxol) also undergo mitotic arrest and enter apoptosis rapidly, and directly, from mitosis [29]. Since these drugs are thought to trigger the spindle assembly checkpoint which in turn acts by inhibiting the APC/C [30-32], it is possible that loss of APC/C activity is responsible for triggering apoptosis. Inactivation of the APC/C by cleavage of the CDC27 component by caspases has also been shown to occur during apoptosis triggered by Fas ligand in Jurkat cells, contributing to an increase in Cdk activity [33]. There have been several reports of increased Cdk activity during apoptosis [34-36], suggesting that these enzymes form part of the apoptotic pathway. Increased mitotic cyclin levels, and Cdk activity, may therefore play a role in apoptosis triggered by loss of APC/C function. Apoptosis, however, does not normally occur when cyclin levels are high at metaphase. This may be because of protective factors such as survivin [27,28]. A loss of protective activity during anaphase may allow cells to respond to abnormally high levels of Cdk activity and undergo apoptosis. Alternatively, if the APC/C itself plays a protective role, simultaneous loss of this protection and elevated Cdk levels would result in apoptosis.

The polyploid cells we observed in larval brain squashes may be cells that have escaped apoptosis, exited mitosis without cytokinesis, and then duplicated their chromosomes before re-entering mitosis again. If so, some cells can clearly repeat the process several times, as we observed cells that were highly polyploid. Furthermore, we did not observe any G2-arrested interphase larval abdominal histoblasts undergoing apoptosis (data not shown). This suggests that there is a phase, during mitosis, when cells are particularly sensitive to loss of lmg function and respond by undergoing apoptosis. This might be expected if loss of APC/C function is playing a relatively direct role in triggering apoptosis.
Conclusions
The data presented in this paper demonstrate that the upstream member of a dicistronic gene, lmgA codes for the Apc11 subunit of the APC/C in a multicellular metazoan species, Drosophila melanogaster. Its genetic and physical interactions with Mr/Apc2 and the E2-C type ubiquitin-conjugating enzyme, Vihar, suggest that their ternary complex represents the same catalytic module of the APC/C that was identified in yeast and mammalian cells by functional means.

Methods
Yeast strains and techniques
Standard yeast media and yeast techniques were used [37]. S. cerevisiae l40 strain (MATa ade2 his3Δ200 leu2-3,112 trp1Δ1 ura3::lexAop-lacZ lys2::lexAop-HIS3) was used for two hybrid, and the Tat7 strain (MATA ura3-52 ade2 his3Δ200 leu2-3,112 trp1Δ1 ura3::lexAop-lacZ lys2::lexAop-HIS3, generously provided by Jacques Camonis) for three hybrid analysis. W303 (MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3) and an APC11-myC9 strain (MATa ApcC11myC9-TPr1 ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura3, kindly provided by Wolfgang Zachariae) were used for the heterologous complementation experiments.

Yeast two and three hybrid experiments
A Drosophila embryonic cDNA library cloned into the pACT vector (Clontech Laboratories, Inc., USA) was screened using apc2-pBTM116 as bait as described by the manufacturer (Clontech Laboratories, Inc., USA). The lmgA and lmgB sequences were cloned into pGAD424 and pACT2 respectively, and then co-transformed with apc2-pBTM116 into L40 strains to confirm their interaction. The cDNA library was also screened with the lmgB-pBTM116 clone as bait. Yeast three hybrid analyses were performed using lmgA-pBTM116, apc2-pRS416 (kindly provided by Jacques Camonis) and vihar-pGAD424 which were co-transformed into the Tat7 strain. His+ colonies were selected and tested for their β-galactosidase activity.

β-galactosidase assay
Colony-lift filter assays were performed as described in the Yeast Protocols Handbook (Clontech Laboratories, Inc., USA). For quantification of β-galactosidase activity, single colonies were grown until mid-log phase in liquid minimal medium containing 1 mM 3-Amino-1,2,4-triazole, and in the presence or absence of 1 mM methionine. Liquid cultures were used for Pellet X-Gal (PXG) assay [38] and for Beta-Glo Assay according to the manufacturer's protocol (Promega Corporation, USA).

Yeast heterologous complementation test
The lmgA cistron was cloned into the pRS426 yeast expression vector (kindly provided by Ildikő Unk). As a control the mks/Apc3 gene was also cloned into pRS426. APC11-myC9 temperature sensitive mutant cells [11] were transformed with lmgA-pRS426, mks-pRS426 and empty pRS426 plasmids. Single Ura+ colonies were selected on minimal medium then inoculated into 3 ml YEPD (BIO 101, Inc., Canada) media and incubated overnight at 30°C in a water bath. Overnight cultures were diluted to OD600 = 0.1, and divided into 6 glass tubes, 3 ml each. 3 tubes were incubated at 30°C and 3 tubes at 37°C in water baths for 10 hours. The W303 strain was used as a control. Optical density was measured every hour using a WPA Biowave CO8000 Cell Density Meter. Data were analyzed and densitometric growth curves were made using Microsoft Office Excel™. For the colony forming dilution, single colonies were inoculated and grown overnight at 30°C. Cultures were diluted to OD600 = 0.1 and then two-fold serially diluted five times. 5 µl of each dilution was spotted onto YEPD plates which were incubated at 30°C and 37°C for 1 day.

Drosophila stocks and genetic techniques
Fly stocks were reared on standard yeast/dextrose medium at 25°C. Stocks were obtained from the Bloomington Drosophila Stock Center. In all experiments a w1118 isogenic stock was used as the control. All genetic markers used are described in Flybase http://flybase.org. To determine the lethal phase, chromosomes carrying the mutations were balanced over CyO, actGFP or TM6C, Tb, Sb chromosomes. From each line 20-30 pairs of flies were placed into chambers on agar plates containing yeast extract. 400-600 first instar homozygous and heterozygous larvae were collected and put into vials, 50-50 each. Metamorphosis was staged according to Bainbridge and Bownes [15].

P-element remobilization
Originally, both the P{PZ}03424 and P{EPgy2}EY11317 mutant stocks carried second site mutations which were removed by recombination before experimental use. Imprecise excisions of the P{PZ}03424 and P{EPgy2} EY11317 elements were generated by crossing to flies carrying the Δ2-3 transposase. Chromosomes which had lost the ry+ or w+ genes in the P element were selected and balanced over CyO, actGFP. For deletion mutant screening, genomic DNA was extracted from candidate lines as by Gloor et al [39] and PCR was performed using the primers specific to CTCCCAGCAAGGTAGTCGA-TATCTTT-3' and 5'-TATGGTGGGGATTTGTGTGTAATG-3' primers. The P{PZ}03424 remobilization
generated the *lmg<sup>1023</sup>* allele that carries a 1543 bp deletion, while the *P[EpGy2]EY11317* imprecise excision yielded two independent lines carrying deletions in the *lmg* gene. In this study we used the *lmg<sup>138</sup>* null mutant which carries an 1120 bp deletion downstream from the P-element insertion site.

**Rescue of the *lmg* mutant phenotype**

Dicistronic *lmgA* and *lmgB* cistrons alone were cloned into the pUAST vector. The resulting plasmids were sequenced and injected into *w<sup>1118</sup>* embryos according to standard protocols [40]. Homozygous transgenic flies carrying one of the *lmgA*-pUAST, *lmgB*-pUAST and *lmgAB*-pUAST constructs on the third chromosome were crossed to *lmg*/*lmg* flies and resulting progeny of *w/w; lmgX/CyO, actGFP; Y*-*pUAST/da-GAL4* in which X represent one of the *lmg* alleles and Y stands for the different *lmg*- pUAST constructs in all combinations. To express *lmg* in these constructs, *w/w; lmg<sup>1118</sup>/CyO, actGFP; Y-*pUAST* flies were crossed to *w/w; lmg<sup>1</sup>/CyO, actGFP; da-GAL4/da-GAL4* flies and resulting progeny of *w/w; lmg/lmg; pUAST*/da-*GAL4* were tested for lethal and mitotic phenotypes.

**Semi-quantitative RT-PCR**

Total RNA was isolated using a Tri Reagent extraction kit (Sigma-Aldrich, USA). RNA samples were treated with RQ1 RNase-Free DNase (Promega Corporation, USA). Reverse transcription was carried out using a Fermentas cDNA synthesis kit using 5 μg RNA and random hexamer primers. cDNA amounts were normalized in 20 cycle PCR using *rpL17A* primers (*rpL17A* upper, 5'-GTGAT-GAACTGTGCCGACAA-3'; *rpL17A* lower, 5'-CCTTCA-TTTCGCCC TTGTTG-3'). PCR products were separated by electrophoresis in a 1.2% agarose gel. To analyze the expression pattern of the *lmg* locus during development, semi-quantitative RT-PCRs were performed. Total RNA was isolated from 50 mg *w<sup>1118</sup>* 0-12 hours embryos, L1, L2, L3, early L3 larvae, early pupae, late pupae, males and females. The following primers were used in 25 cycle PCRs: ORF1 RT primers: 5'-GCCAACCGGGGCACACACGC-3' upstream and 5'-CAGCTCTGGGCGCACAT-3' downstream; ORF2 RT primers: 5'-AATGATGGAGAACAGCAAGGACGATGACAAGTAG-3'; 5'-CCGGCTTGTTGGGTTGATG-3' downstream; Dicistronic RT primers: 5'-CAGCTCTGGGCGCAGAC-3' upstream and 5'-CTTGGCGACAGC-3' downstream. The ORF1 mRNA, PCR was carried out with specific primers for ORF1 (5'-TCCGGGCCAGGTGCTCTCG-3' inner and 5'-CAGCTCTGGGCGGACAT-3' outer) and ORF2 (5'-TCTGGCGACATGGGCGGACTGTGC-3' outer). To obtain the sequence of the 3' end of the ORF1 mRNA, PCR was carried out with specific primers for ORF1 (5'-GCCAACCGGGGCACACACGC-3' outer and 5'-CCGGCTTGTTGGGTTGATG-3' inner). All PCR products were cloned into pTZ57R (Fermentas, Vilnius, Lithuania) and sequenced.

**Epitope construct and Western Blots**

To test the expression of the two *lmg* ORFs, an N-terminal Hemagglutinin tag was attached to ORF1, and a C-terminal Flag tag was made for ORF2. The 5'-TCTGAGATCAAAAGACGAGGGAAT-3' and 5'-CTACTTGTCGCCCCCTTGATTG-3' oligonucleotides were ligated into the *EcoRV*-Xhol sites of pENTRA1, and transformed into DB3.1 competent cells. The *lmgAB* sequence was then ligated into the pENTRA1-Flag tagged construct. The pENTRA1-*lmgA*-Flag was recombined into the pAHW destination vector, which contains three HA epitope tags 5' in its Gateway cassette, using Gateway LR clonase II Enzyme Mix (Invitrogen Corporation, USA). Recombinant clones were selected by ampicillin resistance and sequenced before transfection.

**Cytochrome analysis and Immunohistochemistry**

Orcein staining of larval brain preparations was carried out and analyzed as described previously [14]. Preparations were examined under an Olympus BX51 microscope using phase contrast. Photos were taken by a DP70 digital color camera. Mitotic index was determined as the number of cells in mitosis in an optical field. Apoptotic index was defined as the number of rounded cells with picnotic nuclei in an optical field. Apoptosis was also analyzed by acridine orange staining of dissected brains and wing imaginal discs. Heads of third instar larvae were removed in PBS and transferred into a drop of 1.6 μg/μL acridine orange solution (C.I. 46005 Molar Chemicals Ltd) for five minutes in the dark, then rinsed in PBS. Brains and wing imaginal discs were then dissected and transferred to a drop of PBS on a microscope slide. Before transferring, two cellotape cushions were made on the slide to prevent excess compression. Preparations were covered with a cover-slip, sealed with nail-polish and examined under an Olympus BX51 upright microscope, or with an Olympus FV 1000 confocal microscope.

For immunohistochemistry, brains were dissected from *w<sup>1118</sup>* and *lmg<sup>1023</sup>* wandering third larval instars. Immunostaining preparations, primary and secondary antibodies and image analysis were performed as described in Pál et al. [14].

**RACE Experiments**

Rapid Amplification of cDNA ends was performed using a First-Choice™ RLM-RACE kit (Ambion, USA) according to the protocol provided by the manufacturer. Total RNA was isolated from 50 mg 0-12 hour embryos with Tri Reagent (Sigma-Aldrich, USA). To obtain the 5' end of mRNAs gene specific primers were designed to ORF1 (5'-TCCGGGCCAGGTGCTCTCG-3' inner and 5'-CAGCTCTGGGCGGACAT-3' outer) and ORF2 (5'-TCTGGCGACATTGGGCGGACTGTGC-3' outer). To obtain the sequence of the 3' end of the ORF1 mRNA, PCR was carried out with specific primers for ORF1 (5'-GCCAACCGGGGCACACACGC-3' outer and 5'-CCGGCTTGTTGGGTTGATG-3' inner). All PCR products were cloned into pTZ57R (Fermentas, Vilnius, Lithuania) and sequenced.
Schneider 2 (S2) cells were transfected with the FLAG-LmgAB-HA plasmid using Cellfectin (Invitrogen Corporation, USA) in serum-free S2 cell medium. Total protein was extracted from transfected cells using standard protocols [41] and analyzed by Western blots using anti-Flag and anti-HA monoclonal antibodies.

Acknowledgements

We would like to thank J. Camonis for the generous donation of the Tat7 yeast strain and the pSh416 plasmid, and W. Zachariae for kindly providing the temperature sensitive APC11-myc9 yeast strain. We also appreciate the help of I. Unk, for presenting the pSh426 plasmid to us. We are grateful to our colleagues, L. Kovács for assistance in microscopic imaging and for helpful discussions. ADS thanks A. Spadling for the opportunity to screen the BDGP collection of P element mutants in his laboratory. This work was supported by a Hungarian Scientific Research Fund (OTKA) grant awarded to PD (K 68784) and by a North West Cancer Research Fund grant awarded to ADS.

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ON carried out the genetic, molecular and cytological characterizations of lmgC mutants and isolated the lmg null allele. MP participated in the yeast two- and three-hybrid experiments and in immunohistochemistry. AU carried out the construction and expression of tagged protein expression constructs. CAMS carried out analysis of the APC11-promoterring complex or cyclosome: molecular and genetic characterization of the APC2 subunit, Plant Cell 2003, 15:2370-2382.

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Cite this article as: Nagy et al.: *lemmingA* encodes the Apc11 subunit of the APC/C in *Drosophila melanogaster* that forms a ternary complex with the E2-C type ubiquitin conjugating enzyme, Vilhar and Morula/Apc2. *Cell Division* 2012 7:9.