Lilliputian: an AF4/FMR2-related protein that controls cell identity and cell growth

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SUMMARY

Members of the AF4/FMR2 family of nuclear proteins are involved in human diseases such as acute lymphoblastic leukemia and mental retardation. Here we report the identification and characterization of the Drosophila lilliputian (lilli) gene, which encodes a nuclear protein related to mammalian AF4 and FMR2. Mutations in lilli suppress excessive neuronal differentiation in response to a constitutively active form of Raf in the eye. In the wild type, Lilli has a partially redundant function in the Ras/MAPK pathway in differentiation but it is essential for normal growth. Loss of Lilli function causes an autonomous reduction in cell size and partially suppresses the increased growth associated with loss of PTEN function. These results suggest that Lilli acts in parallel with the Ras/MAPK and the PI3K/PKB pathways in the control of cell identity and cellular growth.

Key words: AF4/FMR2, Cell size, Differentiation, Drosophila melanogaster, PTEN, lilliputian (lilli)

INTRODUCTION

In the development of multicellular organisms, growth and differentiation are tightly coordinated. Although significant progress has been made in the understanding of these two processes individually, we know relatively little about how differentiation is integrated with cellular growth. The first step towards differentiation, the specification of cell fate or tissue types, frequently depends on cellular interactions mediated by conserved signaling pathways. One of the best understood intracellular signaling pathways involves the activation of the small G-protein Ras in response to the stimulation of growth factor receptors including the receptor tyrosine kinase (RTK) family (for review see Boguski and McCormick, 1993; Rommel and Hafen, 1998; Frame and Balmain, 2000). Activation of Ras triggers a highly conserved cascade of kinases including Raf, MEK and MAP kinase (MAPK). In the Drosophila eye, Ras signaling is required for cell growth, survival of postmitotic cells (Bergmann et al., 1998; Dominguez et al., 1998, Kurada and White, 1998), and the specification of ommatidial cell fates (reviewed by Freeman, 1997). Studies in the Drosophila wing have shown that loss of Ras function reduces cell size (Diaz-Benjumea and Hafen, 1994). In the absence of Ras, cell cycle length is prolonged and cells accumulate at the G1/S transition (Prober and Edgar, 2000).

The role of Ras in growth control is shared with the PI3-Kinase/Protein Kinase B (PI3K/PKB) pathway (Oldham et al., 2000a; Weinkove and Leevens, 2000). Cells lacking components of the PI3K/PKB pathway grow more slowly and divide at a smaller size. Neither partial nor complete loss-of-function mutations in genes encoding components of this pathway affect the differentiation or patterning of adult structures. In mammalian cells, the Ras/MAPK and PI3K/PKB pathways cooperate in the control of cell cycle progression. Transition from G1 into S phase of the cell cycle requires the activation of both pathways (Gille and Downward, 1999; Jones et al., 1999). Little is known, however, about where and how these two pathways converge to regulate growth. In this paper, we present evidence that the product of the lilliputian (lilli) gene cooperates with both the Ras/MAPK and the PI3K/PKB pathway in Drosophila in the control of cell fate and cell size.

Mutations in lilli were identified in a screen for dominant suppressors of the rough eye phenotype caused by constitutive activation of Raf during eye development (Dickson et al., 1996). Here we show that Lilli plays a partially redundant function downstream of Raf in cell fate specification in the developing eye. In addition, complete loss of Lilli function leads to a reduction in cell and organ size. The lilli gene encodes a nuclear protein related to the AF4/FMR2 family. In humans, mutations affecting genes of this family are associated with specific diseases. Loss of FMR2 gene transcription causes mental retardation (Chakrabarti et al., 1996; Gecz et al., 1996; Gu et al., 1996). Translocations between MLL (a human trithorax-related gene) and AF4 or AF5q31 are involved in acute lymphoblastic leukemia (Domer et al., 1993; Nakamura et al., 1993; Taki et al., 1999, and references therein). Our results provide the first insight into the function of this class of transcription factors during Drosophila development.
MATERIALS AND METHODS

Genetics
13 EMS-induced alleles and three P-element alleles (lilli1 = k05431, lilliP2 = k07920, lilliP2 = k16124; Torok et al., 1993) of the Su(Raf)/2Alilli complementation group were isolated (Dickson et al., 1996). lilli was mapped to the cytological interval 23C1-2 by combination mapping (Dickson et al., 1996) and by non-complementation with the deficiencies Df(2L)C144 and Df(2L)JS17. The chico1 allele and the P1TEN133 alleles have been described previously (Boehni et al., 1999; Oldham et al., 2000b). The ey-FLP system has been described by Newsome (Newsome et al., 2000).

Clonal analysis
Different lilli alleles were recombined onto FRT40 chromosomes and clonal analysis was performed using the FLP/FRT system described by Xu and Rubin (1993). For the generation of clones in the adult eye, larvae of the genotype y w hs-Flp; lilli FRT40 P(w+) FRT40 were subjected to a heat shock 24-48 hours after egg deposition (AED) for 1 hour at 37°C to induce mitotic recombination. Histological sections of the eyes were done as described previously (Basler and Hafen, 1988). To generate clones in the imaginal discs, larvae of the genotype y w hs-Fly/w; lilli FRT40 P(arm-lacZ w+) FRT40 were subjected to a heat shock 48-72 hours AED for 25 minutes at 33°C to induce mitotic recombination at a low frequency. Larvae at late third instar stage were dissected. Discs were fixed and permeabilised and stained with antibodies. Antibodies were: mouse anti-β-gal (1/2000, Promega), rabbit anti-phosphohistone H3 (1:2000, Upstate biotechnology) and FITC-secondary antibodies (1/200, Jackson ImmunoResearch). Nuclei were stained using the TO-PRO3 DNA stain (T3605, Molecular Probes). Pictures were taken on a Leica TCS-NT confocal laser scanning microscope and clone size was measured by using the NIH image program. The TUNEL assay was performed as described by Boehni et al. (Boehni et al., 1999).

Cloning of lilli
Genomic DNA fragments flanking each of the three P-element insertions were cloned by plasmid rescue (Modzik et al., 1990) and sequenced. This allowed us to locate the lilli gene on contig AC007765 of the Berkeley Drosophila Genome Project (BDGP) which is consistent with the previous cytological localization of lilli to the interval 23C1-2. Overlapping partial lilli cDNAs were used to reconstruct the full length cDNA sequence. This sequence data has been submitted to the GenBank database under accession number AF293971. cDNA clones were isolated from a λgt10 third instar eye-antennal disc library and by searching the BDGP database. The longest cDNAs identified were LD15159, GH23691 and LD18077. LD15159 contains the sequence of exons 1, 2 and the first part of exon 3, while GH23691 and LD18077 contain the cDNA sequence of the 3' part of lilli starting within exon 7. LD18077 had a much longer 3' UTR than GH23691. A polyadenylation signal was identified only in the 3' UTR of LD18077, but not of GH23691, suggesting that the latter cDNA corresponds to a truncated message.

The Genespan program (Burge and Karlin, 1997) predicted the 3' end of exon 3 and the 5' end of exon 7 and in addition exons 4-6 that were not present in the isolated cDNAs. The missing part of the lilli cDNA sequence was isolated by reverse transcriptase-PCR (RT-PCR). RNA from Drosophila embryos and larvae was isolated and used to synthesize first strand cDNA with random or oligo-dT primers. PCR was performed by using different combinations of primers, that matched the sequences of cDNAs LD15159 and GH23691 or of the predicted exons 4-6. We were able to amplify and sequence partial overlapping cDNAs spanning exons 2 and 3 (with primers LD5 5'-TACATAFACCAACATCGCCG-3' and FW6 5'-GATGTGTCATCTTTGCAGC-3'), exon 3 to exon 5 (with primers ofFW22 5'-CCTCGACCTTAACCCTTTGG-3' and oFW24 5'-GTGACTCCGGAGCTGCTTTG-3'), and exon 5 to exon 7 (with primers ofFW23 5'-AGGCAGCCAAATGCCTTG 3' and of FW26 5'-CCGCTCACTTCTCGT)](3')].

In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989). Antisense RNA probes were generated with the DIG RNA labeling kit (Boehringer Mannheim) using cDNA LD18077 as a template. Sense probes generated in parallel with the same template were used as negative controls.

Molecular characterization of lilli alleles
Genomic DNA from flies heterozygous for lilli was isolated. DNA fragments corresponding to exons 3-12 were amplified by PCR and either sequenced directly or subcloned, using the pCRII-Topo cloning kit (Invitrogen), before sequencing. PCR-induced mutations were excluded by sequencing several independent PCR products for each allele.

Generation of flies expressing lilli transgenes
To generate the UAS-lilli rescue construct a 4.4 kb genomic fragment containing the coding part of exon 3 and ending at the Nhel site within exon 7, was fused upstream of the lilli cDNA GH23691 (GH23691 fragment starting with Nhel site in exon 7 and ending with exon 12; Fig. 2A). This fusion gene was subcloned into the pUAST Drosophila transformation vector (Brand and Perrimon, 1993) to generate UAS-lilli (constructed pWF13). The HA-tag was introduced at the 3' end of the open reading frame in UAS-lilli using pMzS5 (a gift from M. Zecca and D. Nellen) to generate UAS-lilli-HA (constructed pWF9). The UAS-lilli construct was expressed by using the UAS-GAL4 system (Brand and Perrimon, 1993). The driver lines were hs-GAL4 (obtained from the Bloomington stock center) and sep-S-P-GAL4, which expresses GAL4 under control of the sev-enhancer-sev-promoter regulatory elements in R7 precursor cells (P. Maier and E. H., unpublished results).

Subcellular localization of Lilli-HA was analyzed in third instar larvae of the genotype y w UAS-lilli-HA/+; hs-GAL4/+ . Salivary glands were dissected 90 minutes after heat shocking for 1 hour at 37°C. They were fixed, permeabilised and stained with the mouse anti-HA antibodies (1/1500, Boehringer Mannheim) and FITC secondary antibodies (1/60). Pictures were taken using a Leica TCS-NT confocal laser scanning microscope. For generating marked clones overexpressing the UAS-lilli rescue construct transgenic flies were used containing the GMR regulatory sequence (Hay et al., 1997) and the GAL4 gene separated by a flip-out cassette containing the w* minigene as a marker (F. Rintelen and E. H., unpublished results). In heat shocked larvae of the genotype y w hs-Fly/w; GMR>FRT white* FRT>GAL4/UAS-lilli the w* cassette is removed in clones thus permitting expression of UAS-lilli under the control of the GMR-GAL4 driver.

RESULTS

Lillii has a redundant function in photoreceptor cell fate specification
Mutations in lilli were identified in two independent genetic screens aimed at identifying novel components in the Ras/MAPK pathway that regulates R7 photoreceptor development. Dickson et al. (Dickson et al., 1996) recovered multiple alleles of a complementation group called Su(Raf)/2A in a screen for mutations that dominantly suppress the multiple-R7 phenotype resulting from constitutive activation of the Raf kinase. Neufeld et al. (Neufeld et al., 1998) recovered alleles of a complementation group called Ss2-1 in a screen for mutations that suppress the rough eye phenotype caused by overexpression of Sina. Both Su(Raf)/2A and Ss2-1 were mapped to the same chromosomal location (23C1-2) and...
Mutations in \textit{lilli} affect growth and cell size

Although clones homozygous for \textit{lilli} alleles in the adult eye were wild-type with respect to photoreceptor cell differentiation and arrangement, we noted that in clones of strong alleles (i.e. \textit{lilli}^{M1} and \textit{lilli}^{SD}) the size of the photoreceptor cells was reduced in comparison to the heterozygous cells adjacent to the clone (Fig. 2A). Analysis of mosaic ommatidia showed that this phenotype is cell autonomous. A reduction of cell size in mutant tissue is typical for components of the PI3K/PKB and Ras/MAPK pathways (Oldham et al., 2000a; Weinkove and Leevers, 2000).

In addition to reducing cell size, loss of Lilli function may also decrease cell number. Clones mutant for \textit{chico}, which display similar cell size defects as \textit{lilli}, grow more slowly and cannot compete with faster growing wild-type cells (Boehn et al., 1999). In contrast, two experiments indicate that growth of \textit{lilli} mutant tissue is not impaired during early larval development: first, when we generated sister clones, one homozygous for \textit{lilli}, the other homozygous for \textit{chico}, we found that \textit{lilli} mutant cells competed successfully with heterozygous tissue while \textit{chico} mutant cells were outcompeted (Fig. 2B). Second, the size of \textit{lilli} clones was similar to that of their wild-type sister clones in the wing imaginal disc and the eye imaginal disc anterior to the furrow.

Posterior of the furrow however, \textit{lilli} mutant clones where significantly smaller than their wild-type sister clones (Fig. 2C,D). Cells posterior to the morphogenetic furrow undergo a final round of cell division and are then integrated into the ommatidial clusters (Freeman, 1997). The reduced clone size could thus be the result of a failure of some \textit{lilli} mutant cells to undergo this last division or to a slight increase in apoptosis in \textit{lilli} clones posterior to the morphogenetic furrow. Using TUNEL staining to detect cells undergoing apoptosis, and anti-
phosphohistone H3 staining to detect mitotic cells, we were unable to observe a significant difference between mutant and control clones. The relatively small difference in clone size may make it difficult to observe significant differences in cell death or mitosis with methods that detect cells undergoing cell death or cell division, respectively only at the time of the experiment. Therefore, at present we do not know whether the reduction in clone size posterior to the morphogenetic furrow is due to an increase in apoptosis or an inhibition of the cell cycle or a combination of both.

Fig. 2. Lilli function is also required for cell and organ size but not for cell proliferation. (A) Tangential section through an eye containing a lilli4U5 clone (marked by the absence of red pigment). Within the clone, all photoreceptor cells are reduced in size compared to the neighboring wild-type and heterozygous photoreceptor cells. At the border of the clone, mosaic ommatidia composed of phenotypically wild-type photoreceptors (marked by the faint yellowish pigment at the base of their rhabdomeres) and small mutant photoreceptor cells are visible, indicating that lilli controls cell size autonomously. Some additional small lilli4U5 clones consisting of only few cells are visible dispersed in the area of wild-type cells. (B) Cell competition assay. lilliP1 homozygous mutant cells are marked by the dark red pigment because they contain two copies of the w+ marker. Cells heterozygous for lilliP1 and chico1 are orange (w+/w). Cells homozygous for chico1 are marked by the absence of pigment (w/w) and are not visible in the adult eye because they have been out-competed by the faster growing heterozygous and homozygous lilli cells. (C) Lilli is not required for cell proliferation but lilli clones posterior to the morphogenetic furrow are reduced in size when compared to their sister clones. Confocal image of lilli4U5 mitotic clones that were generated by heat shock 48-72 hours after egg deposition and analyzed in late third instar larvae. Clones of lilli4U5 cells are marked by the absence of anti-β-gal staining (not green). Their corresponding sister clones are marked by the more intense green staining (two copies of lacZ). Nuclei are stained by TO-PRO (red). For two clone/sister clone pairs the border is indicated. The gray bar at the top of the picture indicates the position of the morphogenetic furrow. Anterior is to the left and dorsal is up in A-C. (D) Analysis of growth properties of lilli4U5 clones in comparison with their wild-type sister clones in imaginal discs. The areas of clone/sister clone pairs in the pouch region of the wing imaginal disc, and in the eye imaginal disc either anterior or posterior to the morphogenetic furrow are shown. Graphs show the sizes (clone areas in pixels) of individual pairs of lilli4U5 clones (black bars) and +/+ sister clones (white bars). In addition, the area of the lilli4U5 clone relative to its sister clone was calculated for each pair. The means of these values are (as a percentage of sister clone ± standard deviation): for wing pouch 102±19%, for the eye anterior to the morphogenetic furrow: 83±33%, for the eye posterior to the morphogenetic furrow: 56±21%. Similar results were obtained for the lilli15D1 allele (data not shown). The ratio for clones in the eye posterior to the furrow was significantly smaller than the ratio for clones anterior to the furrow (t-test: P<0.025; 9 ratios of clones anterior to the morphogenetic furrow were compared with 9 ratios of clones posterior to the furrow).

(E) Selective removal of Lilli function from the eye imaginal discs using the ey-Flp system (Newsome et al., 2000) results in heads that are reduced in size (left fly) when compared to wild-type flies (right). Genotypes: (A) y w hs-Flp/y; lilli4U5 FRT40/P(w+) FRT40; (B) y w ey-Flp/Y; lilliP1 FRT40/chico1FRT40; (C) y w hs-Flp/y; lilli4U5 FRT40/P-arm-lacZ w+) FRT40. (E, left side) y w ey-Flp/y; lilli4U5 FRT40/P(w+) l(2)2L-3.1 FRT40; (E, right side) y w ey-Flp/y; lilli4U5 FRT40/CyO.
Lilli regulates cell growth and cell differentiation

Consistent with the reduced clone size in the eye disc posterior to the morphogenetic furrow we found that the selective removal of Lilli function in eye and head precursor cells by the ey-Flp system resulted in flies with reduced eye and head size (Fig. 2E). The reduction in eye size was caused by a reduction in both the number and size of ommatidia (24% and 18%, respectively, for lilli4U5; Fig. 3I,K). The degree of head size reduction allowed us to define a class of strong alleles (e.g. lilli4U5, lilli15D1) and a class of weak alleles (e.g. lilli3E8, lilliP1, lilliP4). The small head phenotype of lilli mutant tissue is similar to that observed with components of the PI3K/PKB pathway (Boehni et al., 1999; Montagne et al., 1999).

Lilli partially suppresses the overgrowth phenotype caused by the loss of PTEN function

Since loss of Lilli function affects cell size and head size, we tested for genetic interactions between lilli and components of the PI3K/PKB pathway. PTEN acts as a negative regulator in the PI3K/PKB pathway by dephosphorylating the second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3; PI3P, for reviews see Rameh and Cantley, 1999; Di Cristofano and Pandolfi, 2000). Tissues mutant for PTEN show hyperplastic and hypertrophic growth: PTEN mutant cells are larger and proliferate at a higher rate than wild-type cells (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000).

Removal of PTEN function from the eye imaginal disc tissue using the ey-Flp system (Newsome et al., 2000) resulted in an increase in eye and head size (Fig. 3A; Oldham et al., 2000b). The increase in eye size was due to an increase in cell number and cell size as indicated by the increase in number and size of ommatidia (Fig. 3L,K). To test whether the PTEN large-head phenotype was modified by the removal of Lilli function, we generated PTEN, lilli double mutant eyes. Indeed, eyes double mutant for PTEN and lilli were considerably smaller than PTEN mutant eyes due to a reduction in cell number and cell

Fig. 3. lilli cooperates with PI3K/PKB signaling.

(A-H) Scanning electron micrographs of eyes in which the function of PTEN or Lilli or both has been removed from most of the cells by the ey-Flp system. Images in the same row (i.e. A-D or E-H) were taken at the same magnification. Scale bars 100 μm (A-D) and 20 μm (E-H). For A-D and L, anterior is to the left and dorsal is up. (A,E) Removal of PTEN leads to increased size of the head capsule and the eyes due to hyperplastic growth. In addition, the ommatidia are enlarged and additional bristles are formed. (B,F) Heads double mutant for both PTEN and lilli are smaller than those of PTEN single mutants or wild type. Interommatidial bristles are lost in lilli, PTEN double mutant heads. (C,G) lilli mutant heads are smaller than wild-type heads and have ommatidia of reduced size. (D,H) Control flies in which the ey-Flp system is used in combination with an FRT40 chromosome alone (i.e. without a mutation). The size of the head is nearly wild type. (I) Number of ommatidia in eyes of the same genotype as shown in A-H. Scanning electron micrographs of female flies of the indicated genotypes were taken and used to count ommatidia. The standard deviation is indicated by the error bar. The differences between the mean values is significant (t-test: P<0.025, n=4 eyes for all genotypes). (K) Size of ommatidia in eyes of the same genotype as shown in A-H. Scanning electron micrographs of female flies of the genotypes indicated were used to determine the average area covered by an ommatidium, based on measurements of 7 neighboring ommatidia. The mean values are significantly different except for the difference between ‘PTEN+lilli’ and ‘control’ (t-test: P<0.025, n=4 eyes for each genotype). (L) hs-Flp-induced clone in the eye lacking both Lilli and PTEN function. The clone is marked by the absence of red (w+) pigment. One mutant photoreceptor (no red pigment in rhabdomere) and a heterozygous photoreceptor (pigment present in rhabdomere) are marked by a black or a white arrow, respectively. Genotypes: (A,E: ‘PTEN’): y w ey-Flp/w; PTEN117.35 FRT40/P(w+) l(2)2L-3.1 FRT40; (B,F: ‘PTEN+lilli’) y w ey-Flp/w; lilli4U5 PTEN117.35 FRT40/P(w+) l(2)2L-3.1 FRT40; (B,F: ‘lilli’): y w ey-Flp; lilli4U5 FRT40/P(w+) l(2)2L-3.1 FRT40; (D,H: ‘control’) y w ey-Flp/w; FRT40/P(w+) l(2)2L-3.1 FRT40; (L) y w; hs-Flp/w; lilli4U5 PTEN117.35 FRT40/P(w+) FRT40.
Fig. 4. Molecular organization of the lilli locus and characterization of mutant alleles. (A) Genomic structure of the lilli locus at 23C1-2. The position of the centromere is to the right. Transcription units of the β-subunit of the geranylgeranyl transferase (β-geg), the NTase, and the RNA-binding protein 9 (ebp9) are indicated. For the lilli transcript individual exons are shown. White boxes represent non-coding sequences and black boxes the lilli coding region. The approximate locations of P-element insertions in lilli (P1: lilliP1=p05431, P2: lilliP2=p07920, P4: lilliP4=p16124) are indicated by inverted triangles. Below, the lilli rescue construct is shown (see text for details). (B) Schematic representation of the Lilli protein indicating the predicted domains and sequence similarities to other proteins (for amino acid positions see text). Lilli shares the serine-rich (Ser) and the C-terminal homology domain (CHD) with proteins of the AF4/FMR2 family but does not contain the NHD and ALF domain. Q1-Q4: glutamine-rich stretches. Homology with POU: region homologous to part of the transactivation domain of POU class III transcription factor from chick. AT-hook: AT-hook motif. NLS1 and NLS2: putative bipartite nuclear localization signals predicted by the psfscan program (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Locations of the mutant lesions are indicated above the protein structure. Moleculerly, the mutations consist of the following changes (in brackets: first amino acid affected by the mutation): lilli5X5: CAG (Q192) → TAG (stop); lilli4P5: CGA (R803) → TGA (stop); lilli16G1: 20 bp deletion starting within the codon for T758 which results in the replacement of the original amino acid sequence with a shorter sequence with shifted reading frame; lilli3X5: CAG (Q1610) → TAG (stop); lilli16F1: TAC (Y1459) → TTC (F1461); lilli16Q1: 101 bp deletion, starting within the codon for L1352 which results in the replacement of the original amino acid sequence with a shorter sequence with shifted reading frame; lilli16Q2: CGA (R1461) → TGA (stop); lilli22E1: CAA (Q1690) → TAA (stop). (A) A short amino acid stretch in Lilli is homologous to part of the POU class III transcription factor from chick. The black boxes indicate amino acid identity while the gray boxes indicate amino acid similarity. Dots indicate gaps in the sequence that were introduced to optimize the alignment. (D) Alignment of the AT-hook of Lilli with that of human HMG-I(Y) (Homo sapiens, amino acid 7-34; GenBank accession number L46353) and rat HMG-I(Y) (Rattus norvegicus, amino acid 7-34; GenBank accession number U98695). Gaps and identical or conserved positions are indicated as in C. (E) The HA-tagged version of Lilli (Lilli-HA) is localized in the nucleus when overexpressed in salivary glands. A confocal image of salivary glands stained with anti-HA is shown. Lilli-HA expression was induced by heat shock in vitro (Ma and Staudt, 1996; Gu et al., 1996; Nilson et al., 1997) homologous to members of the AF4/FMR2 family of mammalian proteins (Fig. 4B,F). In this region, Lilli is 31-37% identical to the corresponding sequences of human AF4, FMR2, AF5q13 and LAF4. Interestingly, the position of the intron/exon boundaries within the region encoding the CHD domain are conserved between lilli and the AF4 family members suggesting that those exons have a common evolutionary origin.

Searching the Drosophila genome database did not reveal any sequences in addition to lilli that share homologies with AF4/FMR2 family members. Thus lilli appears to be the only member of this family in Drosophila. FMR2, AF4 and LAF4 are nuclear proteins and these have properties of transcription factors. They possess a putative transactivation domain and have the ability to bind DNA in vitro (Ma and Staudt, 1996; Gu et al., 1996; Nilson et al., 1997; Gecz et al., 1997; Miller et al., 2000). Like other family members, Lilli is rich in proline and serine residues (9.0% and 12.7% of all amino acids, respectively). For human AF4 and LAF4, the serine-rich region has been shown to be situated in a part of the protein that has transactivation potential (Ma and Staudt, 1996). Serines are concentrated in Lilli at the same relative position as in AF4/FMR2 family members (Ser domain, amino acid 860-965 of Lilli; Fig. 4B). However, domains within the AF4/FMR2 family members that are located N-terminal of the serine-rich region (i.e. NHD domain and ALF domain, defined by Nilson et al., 1997) are not present in Lilli.

In addition to the serine-rich region, two other features of Lilli indicate a possible function in activating transcription: first, four glutamine-rich stretches in the N-terminal region of Lilli (Q1-Q4: amino acids 3-14, 32-48, 172-180 and 500-521) initiate a genomic walk of the 23C1-2 region. A search for cDNA clones in this region revealed several ESTs that consisted of exons located near the insertion site of the P elements. Sequencing of all available cDNAs defined a transcription unit spanning a region of 67 kb and consisting of 12 exons (Fig. 4A). Since one P element is located in the first intron and two other P elements are inserted in the second 54 kb intron, this transcription unit was a good candidate for the lilli gene. Two further findings demonstrate that this candidate gene is indeed responsible for Lilli function. First, missense or nonsense mutations were identified in the predicted lilli transcription unit in 8 out of 11 EMS-induced lilli alleles examined (Fig. 4B, and see below). Second, low level ubiquitous expression of a minigene rescue construct (UAS-lilli in Fig. 4A) using a heat-shock-GAL4 driver in the absence of heat shock rescued lilli homozygous mutant flies to adulthood. The rescued flies appeared normal – except that the size of the wing and the head was slightly reduced in comparison to wild-type flies (data not shown). Furthermore, the dominant suppression of the s-E-Ras-pov19-induced rough eye phenotype by removal of one copy of lilli was reverted by expressing the UAS-lilli minigene under the control of the sevE-sevP-GAL4 driver (data not shown). These results establish the identity of the lilli gene.

**lilli encodes a novel nuclear protein related to the mammalian AF4/FMR2 family of transcription factors**

The predicted lilli open reading frame encodes a protein of 1673 amino acids which has a C-terminal domain (amino acids 1418-1668, C-terminal homology domain, CHD, defined by Nilson et al., 1997) homologous to members of the AF4/FMR2 family of mammalian proteins (Fig. 4B,F). In this region, Lilli is 31-37% identical to the corresponding sequences of human AF4, FMR2, AF5q13 and LAF4. Interestingly, the position of the intron/exon boundaries within the region encoding the CHD domain are conserved between lilli and the AF4 family members suggesting that those exons have a common evolutionary origin.

size (Fig. 3B,C.I,K). Loss of Lilli function, however, did not completely suppress the PTEN phenotype. Although these results suggest that Lilli and PTEN cooperate in the control of cell and organ growth, the absence of a clear-cut epistasis between the two mutants indicates that Lilli does not act downstream of PTEN in a simple linear path way but it rather acts in a parallel pathway required for growth.

**Molecular characterization of the lilli gene**

To identify the lilli gene, DNA flanking the lilli P-element insertions (see Materials and Methods) was cloned and used to...
and, second, a domain (amino acid 245-300) with homology to part of a putative transactivation domain from the POU class III transcription factor (Fig. 4B,C; Levasseur et al., 1998).

Lilli has two putative bipartite nuclear localization signals (amino acid 889-906 and amino acid 1286-1303, respectively, Fig. 4B). In addition, Lilli has a sequence (amino acid 836-859; Fig. 4B,D) which matches the consensus sequence for the HMG-I(Y) DNA binding motif, termed the AT-hook (Reeves and Nissen, 1990). This DNA binding domain is not found in other members of the AF4/FMR2 family. The AT-hook motif binds to the minor groove of the DNA double helix at AT-rich sequences (Reeves and Nissen, 1990).

Analysis of the EMS-induced mutations provided further insight into putative functional domains of the Lilli protein (Fig. 4B and figure legend). lilli4U5 contains a short deletion resulting in a frameshift and is probably a functional null allele, since the encoded protein lacks most of the predicted domains including the AT-hook, the serine-rich domain, and the nuclear localization signals. The lilli3E8 mutant protein is predicted to have a C-terminal truncation but since it still retains the putative DNA-binding and Ser domain it is likely to have residual activity. Interestingly, although lilli3E8 was able to suppress the RafY99 induced rough eye to the same extent as the other EMS alleles or deficiencies for the locus, it showed a less severe reduction in head size in ey-FLP mosaics than lilli4U5 and lilli1SD1 (data not shown). This suggests that the very C-terminal domain of Lilli is essential for its function as a transducer of the Raf signal but is less critical with regard to its growth promoting role. In the protein encoded by lilli66F1, Tyr1459 is replaced by alanine. Intriguingly, Tyr1459 is conserved throughout the AF4/FMR2 family (Fig. 4F; the phylogenetic tree of lilli16F1 in ey-Flp mosaics was weak and similar to the Lilli3E8 phenotype).

**Lilli protein is localized in the nucleus**

lilli expression in the embryo was analyzed by in situ hybridization. We found high levels of lilli mRNA in the unfertilized egg, which is consistent with the maternal requirement for Lilli function (Dickson et al., 1996; Perrimon et al., 1996). In the early embryo and until gastrulation, lilli is expressed uniformly. During later stages of embryogenesis, we found a slightly elevated expression in the nervous system (data not shown). In order to determine the subcellular localization of Lilli, we generated an HA-tagged minigene construct (UAS-lilli-HA, see Material and Methods). UAS-lilli-HA rescued lilli lethality suggesting that this construct encodes a functional Lilli protein (data not shown). Staining salivary glands of larvae in which expression of the HA-tagged Lilli (Lilli-HA) was induced by heat shock, revealed that Lilli-HA is mainly localized in the nucleus (Fig. 4E). Nuclear localization of Lilli-HA was also observed in the eye imaginal disc (data not shown). Therefore, we believe that, like its mammalian homologs, Lilli also normally functions in the nucleus (Nilson et al., 1997; Miller et al., 2000).

**DISCUSSION**

FMR2 and AF4 are the founding members of a family of putative transcriptional activators in humans (Domer et al., 1993; Nakamura et al., 1993; Chakrabarti et al., 1996; Gecz et al., 1996; Gu et al., 1996). Little is known about their normal cellular function, although mutations in the corresponding genes are associated with human diseases such as mental retardation and acute lymphoblastic leukemia. Here we describe the characterization of the first invertebrate member of this family. Lilli cooperates with the Ras/MAPK and the PI3K/AKT pathway in the control of cell growth and cell differentiation.

**Lilli has a partially redundant function downstream of Ras/MAPK signaling in cell fate specification**

Loss-of-function mutations in lilli where identified as dominant suppressors of the specification of supernumerary R7 photoreceptor cells in response to constitutive activation of Raf in the developing eye (Dickson et al., 1996). However, without constitutive activity of the Ras/MAPK pathway, the normal number of photoreceptor cells is specified in each ommatidium in the complete absence of Lilli function. Given the fact that lilli encodes a putative transcription factor, a possible explanation for why lilli mutants specifically suppress the differentiation of extra R7 photoreceptor cells is that lilli may be required for the expression of the sE-RafY99 transgene. Indeed, we and others have noted that lilli mutations affect transcript levels of transgenes containing a hsp70 basal promoter (data not shown and Tang et al., 2001). Although the effect of lilli on hsp70 transcription may explain some of the genetic interactions (Greaves et al., 1999; Rebay et al., 2000), it is not sufficient to explain the specific interaction with the Raf/MAPK pathway for the following reasons. (1) In the original screen, we used two different RafY99 transgenes. One contained the sev enhancer fused to the hsp70 promoter (sE), the other the sev enhancer fused to the sev promoter (sEsP; Dickson et al., 1996). lilli mutations suppressed both. (2) lilli mutations failed to suppress the rough eye phenotype caused by the ectopic expression of rough under the control of the sev enhancer and heat shock promoter. (3) Heterozygosity for lilli does not suppress the multiple R7 phenotype of SevS11, a transgene encoding a truncated version of the Sev receptor also expressed under the control of the sev enhancer and the hsp70 promoter (sE; Basler et al., 1991). The formation of multiple R7 cells caused by sE-Sept11 or sEsPRasS12 (Fortini et al., 1992) is only suppressed in homozygous lilli clones (Dickson et al., 1996). If lilli controlled expression of the sev enhancer, we would expect a dominant suppression of all sev enhancer driven transgenes. (4) Finally, as we show here, complete loss of Lilli function in clones decreased the number of photoreceptor cells recruited in the background of a hypomorphic mutation of raf, rafHMT7. Therefore, we conclude that Lilli has a specific function in regulating the efficiency of signal transduction downstream of Raf. As a putative transcription factor, Lilli may regulate the expression levels of one or multiple components of the Ras/MAPK signaling pathway that become rate-limiting when Ras/MAPK signaling is too high, in cells where it is normally low (as in the case of ectopic activation of Raf in the eye), or when signaling is reduced.

**Lilli function in growth control**

Growth is controlled by many different signals and during development it is tightly linked to pattern formation (Day and Lawrence, 2000). In *Drosophila*, the insulin receptor controls
growth via the PI3K/PKB pathway without affecting pattern formation (reviewed by Oldham et al., 2000b; Weinkove and Leevers, 2000). The EGF receptor also controls growth most likely via the Ras/MAPK pathway (Diaz-Benjumea and Hafen, 1994; Prober and Edgar, 2000). Little is known about the nuclear factor Lilli be a point of convergence of these two pathways for growth control? Although the cell size phenotype observed in lilli mutants is similar to that of mutants in either of the two pathways, mutations in lilli also display some unexpected phenotypes that are difficult to reconcile with Lilli functioning exclusively as a target of the two pathways. (1) lilli mutant cells do not have a growth disadvantage during early imaginal disc development. (2) The partial suppression of the overgrowth phenotype of PTEN mutants by lilli mutants suggests a complex epistatic relationship between lilli and the PI3K/PKB pathway. (3) Both Lilli overexpression and lilli loss-of-function mutations reduce cell size (Fig. 2A, and data not shown). This is in contrast to components of the PI3K/PKB and Ras/MAPK signaling pathways. Overexpression of PI3K or PKB increases cell size, whereas loss-of-function mutations decrease cell size (Leevers et al., 1996; Verdu et al., 1999; Weinkove et al., 1999). Similarly, loss- and gain-of-function mutations of Ras have opposite effects on cell size (Prober and Edgar, 2000). Lilli may activate the transcription of genes involved in cell growth and differentiation.

Lilli and AF4/FMR2 phenotypes
How does the function of Lilli in Drosophila correlate with the phenotypes observed in disease conditions associated with mutations in the human homologs AF4, AF5q31 and FMR2? In the case of acute lymphoblastic leukemia two related genes, AF4 and AF5q31, are involved in translocations to the MLL gene (also known as ALL-1, trtx), which encodes a human homolog of the Drosophila protein Trithorax (Domer et al., 1993; Nakamura et al., 1993; Taki et al., 1999, and references therein). We show that Lilli function is essential for increased activity in the Ras/MAPK pathway. Activating mutations in Ras are frequently found in lymphomas but normally absent in leukemias associated with MLL rearrangements (Mahgoub et al., 1998, and references therein). It is possible that expression of the MLL/AF4 fusion gene circumvents the need for Ras activation in transformation. Recently, the targeted inactivation of the AF4 gene in mice has been reported (Isnard et al., 2000). AF4 mutant mice display an altered development of lymphoid cells and, on a mixed 129/Balb/c background, a subset show a significant reduction in body weight at birth. The reduction in weight may suggest that AF4 and Lilli control cellular growth in mice and Drosophila, respectively. Thus, further analysis of Lilli function may provide insights into the function of this class of nuclear proteins both in vertebrate development and disease.

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