Drosophila S6 Kinase: A Regulator of Cell Size

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Cell proliferation requires cell growth; that is, cells only divide after they reach a critical size. However, the mechanisms by which cells grow and maintain their appropriate size have remained elusive. *Drosophila* deficient in the *S6 kinase* gene (*dS6K*) exhibited an extreme delay in development and a severe reduction in body size. These flies had smaller cells rather than fewer cells. The effect was cell-autonomous, displayed throughout larval development, and distinct from that of ribosomal protein mutants (*Minutes*). Thus, the *dS6K* gene product regulates cell size in a cell-autonomous manner without impinging on cell number.

In unicellular and multicellular organisms, specific cell types have a characteristic size (*1*). In multicellular organisms, the control of cell size has the added importance of dictating the proportions of organs, limbs, and the living entity (*1*). Therefore, mechanisms must exist to integrate cell growth and proliferation, so that cells maintain an appropriate size (*1*). In yeast, a minimum cell size must be achieved before cells initiate S phase and divide, whereas mutations that block cell cycle progression do not inhibit cell growth (*2*). Studies in which cell cycle regulators were manipulated in the imaginal wing disc of *Drosophila melanogaster* (*3*) largely confirmed these observations in metazoans. However, little is known concerning the identity of regulatory components and signaling pathways that control cell growth or the mechanisms by which they are integrated with the control of cell proliferation in the developing organism (*1*, *4*). The mammalian 40*S* ribosomal protein S6 kinases [p70 S6 kinase, also called S6K1, and the very similar S6K2 (*5*)] may represent such regulatory components (*3*). These enzymes serve as the physiological kinases for the ribosomal protein S6 (*5*). Their role in cell growth stems from their function in controlling the increased translation of mRNAs encoding for ribosomal proteins (*6*). In *Drosophila*, a family of mutants defective in ribosomal protein production, termed *Minutes*, display short and slender bristles and a delayed developmental program, which results from a slower rate of cell growth and division (*7*). Because S6K1 regulates ribosomal protein production in mammals, loss of *Drosophila* S6K (*dS6K*) function could have a direct impact on cell growth and proliferation.

We found that a female sterile mutant, *fs(3)07084* (*8*), contained a P-element insertion in the 5′ noncoding region of the *dS6K* gene (Fig. 1A) (*9*). Only 25% of the expected number of homozygous *fs(3)07084* (*dS6K*^{07084}) flies emerged as adults, with a 3-day delay and reduced body size. This phenotype was rescued either by excision of the P element or by a *dS6K* or mammalian S6K transgene (*10*). Northern (RNA) blot analysis (Fig. 1B) (*11*) and sequencing of a reverse transcriptase polymerase chain reaction product (RT-PCR) from homozygous mutant flies revealed the presence of anomalous transcripts (Fig. 1C) (*11*), suggesting that dS6K expression may persist in homozygous *dS6K*^{07084} flies. More severe alleles were generated by imprecise P-element excisions (*12*), removing part of the *dS6K* gene. Most of these flies died as larvae, with the lethality rescued by expression of *dS6K* or mammalian S6K transgenes (*10*). One of the excisions, *dS6K*$^{l\text{-}1}$, removed part of the first exon, including a portion of the catalytic domain (Fig. 1A) (*12*). The few surviving *dS6K*$^{l\text{-}1}$ homozygous flies emerged after a 5-day delay, lived no longer than 2 weeks, and displayed a severe reduction in body size, with all body parts apparently affected to the same extent (Fig. 1D). Thus, loss of dS6K function induces female sterility, a strong developmental delay, a

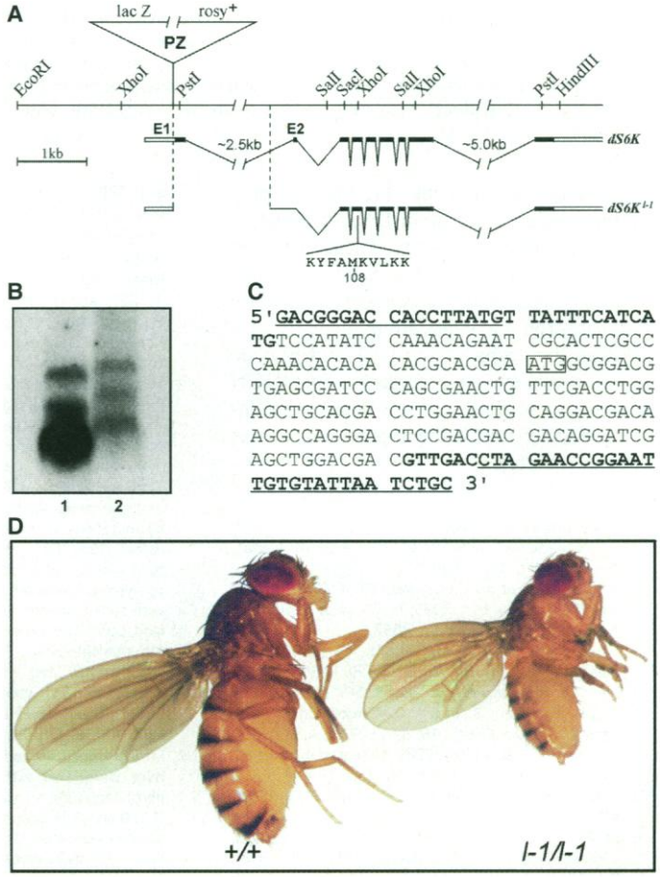

Fig. 1. Identification of P-element–induced mutation in the *dS6K* gene. **(A)** *dS6K* gene showing introns and exons. The *dS6K* cDNA sequence has recently been modified (GenBank accession number U66562). The P-element (PZ) insertion point and the coding sequence (black boxes) are indicated. The mRNA structure of the *dS6K*$^{l\text{-}1}$ deficiency is represented at the bottom. Single-letter amino acid abbreviations are as follows: A, Ala; F, Phe; K, Lys; L, Leu; M, Met; V, Val; and Y, Tyr. **(B)** Northern (RNA) blot analysis with the *dS6K* cDNA as a probe (*11*): (lane 1) three transcripts of 3.3, 4.4, and 5.8 kb detected in total RNA (20 μg) isolated from ovaries of homozygous *dS6K*^{07084} females or (lane 2) three transcripts of 2.8, 3.7, and 5.0 kb in the same amount of total RNA isolated from wild-type females. **(C)** Sequence of RT-PCR fragment generated from mRNA of homozygous *dS6K*^{07084} female ovaries with two oligonucleotides covering part of the P element and the second exon of *dS6K* (*11*). The sequences derived from the P element and the second exon of *dS6K* are in bold. Oligonucleotides used for PCR reaction are underlined, and the ATG translational start codon for *dS6K* is boxed. **(D)** Comparison of wild-type (+/+) and *dS6K*$^{l\text{-}1}$ (l-1/l-1) homozygous females 4 days after emergence. Body weights of wild-type and *dS6K*$^{l\text{-}1}$ homozygous females were 1.49 ± 0.04 mg and 0.80 ± 0.1 mg, respectively.

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severe reduction in growth, and often death.

As mammalian S6Ks control the synthesis of ribosomal proteins (*6*), we hypothesized that the *dS6K*$^{l\text{-}1}$ phenotype might be equivalent to that of *Minutes* (*7*). The *Minute M(3)95A*, harbors a P-element insertion that severely reduces the expression of ribosomal protein S3 (*7*). However, analysis of *M(3)95A* (Fig. 2A) and two other *Minutes* showed no effect on size (*10*), although all displayed a developmental delay and slender bristles. In contrast, the bristles of homozygous *dS6K*$^{l\text{-}1}$ flies were proportional to body size (Fig. 2B). To determine whether the reduction in body size of homozygous *dS6K*$^{l\text{-}1}$ flies was due to a decrease in cell number, we compared cells in wings and ommatidia in eyes of wild-type and *dS6K* mutant flies. The cell density was greater in wings of homozygous *dS6K*$^{l\text{-}1}$ flies (as represented by each hair) than in wild-type flies (Fig. 2C) (*13*). The difference in cell size was almost 30%, and flies homozygous for partial loss of function *dS6K*^{07084} displayed an intermediate cell size (Fig. 2C). However, the total number of cells in wings remained constant (Fig. 2C). Analysis of eyes revealed a similar phenotype with reduced size but no effect on the number of ommatidia (Fig. 2D) (*13*). Thus, in *dS6K* mutants, the decrease in the rate of proliferation (see below) is probably attributable to a reduction in ribosomal protein synthesis, whereas the effect on cell size may be due to the absence of S6 phosphorylation and an altered pattern of translation (*6*).

The reduction in cell size of *dS6K*$^{l\text{-}1}$ flies indicates either that cells are proliferating at a smaller size or that flies emerge from the extensive developmental delay before completion of the last round of cell growth. To examine these possibilities, we analyzed proliferating epithelial cells from the imaginal wing disc of larvae at the end of the third instar (*14*). Imaginal discs give rise to the adult structures (*14*). At the end of the third instar, wing disc cells still require two mitotic cell cycles before they differentiate (*15*). Comparison of wing discs from homozygous *dS6K*$^{l\text{-}1}$ and wild-type larvae revealed that mutant discs were substantially smaller in size (Fig. 3A) (*14*). Analysis of single cells from discs with a fluorescence-activated cell sorter (FACS) confirmed that, on average, cells derived from *dS6K* mutants were smaller than wild-type cells (Fig. 3B). There was no apparent difference between the distributions of *dS6K* mutant and wild-type cells within each phase of the cell cycle (Fig. 3C), implying that the *dS6K*$^{l\text{-}1}$ loss-of-function mutation affected all stages of the cell cycle. Analysis of disc cells during puparium formation, when proportionally more cells are present in G_2 phase (*16*), also showed no detectable difference in the cell cycle distri-

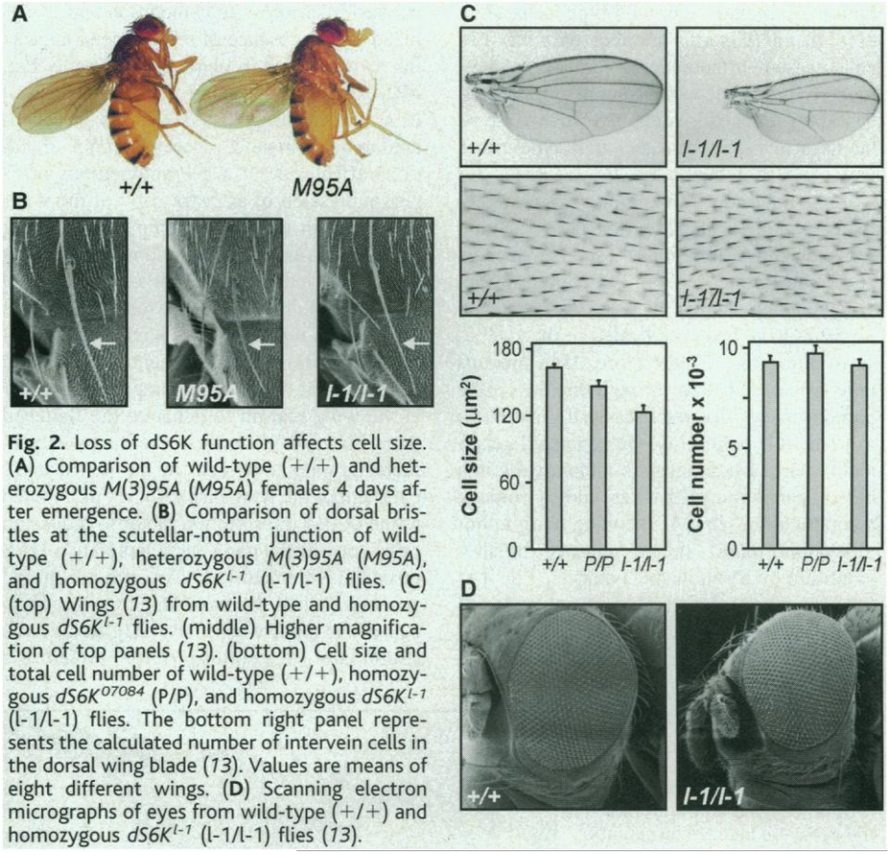

Fig. 2. Loss of dS6K function affects cell size. (**A**) Comparison of wild-type (+/+) and heterozygous *M(3)95A* (*M95A*) females 4 days after emergence. (**B**) Comparison of dorsal bristles at the scutellar-notum junction of wild-type (+/+), heterozygous *M(3)95A* (*M95A*), and homozygous *dS6K*$^{l\text{-}1}$ (l-1/l-1) flies. (**C**) (top) Wings (*13*) from wild-type and homozygous *dS6K*$^{l\text{-}1}$ flies. (middle) Higher magnification of top panels (*13*). (bottom) Cell size and total cell number of wild-type (+/+), homozygous *dS6K*^{07084} (P/P), and homozygous *dS6K*$^{l\text{-}1}$ (l-1/l-1) flies. The bottom right panel represents the calculated number of intervein cells in the dorsal wing blade (*13*). Values are means of eight different wings. (**D**) Scanning electron micrographs of eyes from wild-type (+/+) and homozygous *dS6K*$^{l\text{-}1}$ (l-1/l-1) flies (*13*).

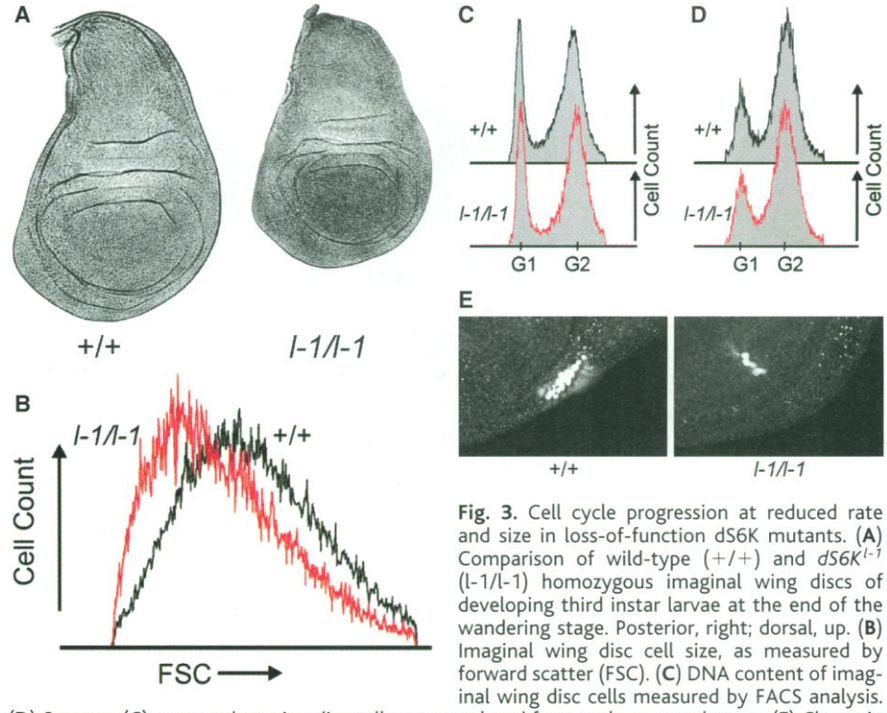

Fig. 3. Cell cycle progression at reduced rate and size in loss-of-function dS6K mutants. (**A**) Comparison of wild-type (+/+) and *dS6K*$^{l\text{-}1}$ (l-1/l-1) homozygous imaginal wing discs of developing third instar larvae at the end of the wandering stage. Posterior, right; dorsal, up. (**B**) Imaginal wing disc cell size, as measured by forward scatter (FSC). (**C**) DNA content of imaginal wing disc cells measured by FACS analysis. (**D**) Same as (C), except that wing disc cells were analyzed from early prepupal stage. (**E**) Clones in wing disc of wild-type (+/+) and *dS6K*$^{l\text{-}1}$ (l-1/l-1) larvae were detected by β-galactosidase antibody staining 53 hours after induction of somatic recombination (*3*). The number of wing disc cells present in 80 clones each of wild-type and mutant larvae was counted, and cell cycle times were determined (*3*).

bution of mutant and wild-type cells (Fig. 3D). In addition, the number of wing disc cells present in somatically induced clones, marked by ectopic expression of β-galactosidase, was reduced in mutant versus wild-type larvae. Consistent with this, cell cycle times were 12.5 ± 1 hours and 24 ± 4 hours for wild-type and mutant wing disc cells, respectively (Fig. 3E). Thus, loss of dS6K function leads to cell proliferation at a smaller size and at a reduced rate, without affecting any specific stage of the cell cycle.

S6Ks have been implicated in the synthesis of mitogens (*17*); therefore, *dS6K* mutants may affect cell size through the loss of a humoral factor that regulates cell growth. To examine this possibility, we generated genetically marked homozygous mutant cells in a heterozygous mutant background by somatic recombination (*18*). At the wing margin, homozygous *dS6K* mutant sensory bristles, identified by a *yellow* (y^-) marker (Fig. 4A) (*13*, *18*), were reduced in size compared with their neighbors. In eyes, homozygous *dS6K* mutant photoreceptor and pigment cells were marked by a *white* (w^-) mutation and recognized by the absence of red pigment, appearing as dark spots in photoreceptor cells (Fig. 4B). Again, only mutant cells were reduced in size, indicating that *dS6K* acts in a cell-autonomous manner. Because *dS6K* mutations affect size in a cell-autonomous manner, expression of an extra copy of the wild-type gene in a specific compartment might positively affect growth. A compartment represents an independent unit of growth and size control, thought to be analogous to a mammalian organ (*1*). The wing disc is composed of a dorsal compartment and a ventral compartment that fold in an apposed manner at the wing margin to generate the flattened wing blade (*19*). Because the *apterous* promoter is only functional in the dorsal compartment of the wing disc (*20*), it was coupled to the GAL4 transcription factor to induce an extra copy of the *dS6K* gene linked to a UAS responsive element (*21*). An increase in cell size of less than 1% should alter the morphology of the adult wing blade (*22*). In all UAS *dS6K* lines we examined, dS6K protein expression was increased and the wing was convex and bent downward (Fig. 4C). The phenotype can be explained by an increase in the size of the dorsal versus the ventral wing surface, forcing the wing blade to curve down to accommodate the greater surface (*22*). Therefore, increased expression of *dS6K* positively affects growth in a cell-autonomous and compartment-dependent manner.

S6Ks appear to be a downstream effectors of the phosphatidylinositide-3OH kinase (PI3K) signaling pathway (*23*). However, activated or dominant interfering alleles of PI3K affect cell number and cell size (*24*). This would imply that S6Ks reside on a branch of the PI3K signaling pathway that controls cell growth and size but not cell number. Overexpression of the cell cycle regulator E2F in the posterior compartment of the wing disc increases cell number without affecting final compartment size (*3*). These findings are consistent with the hypothesis that compartments, like organs, adjust their final mass independent of cell number (*1*). However, the *dS6K* phenotypes described here suggest that cell size participates in the control of compartment size. Indeed, S6Ks are thought to play a critical role in organ hypertrophy, where the organ increases in size as a function of demand (*25*).

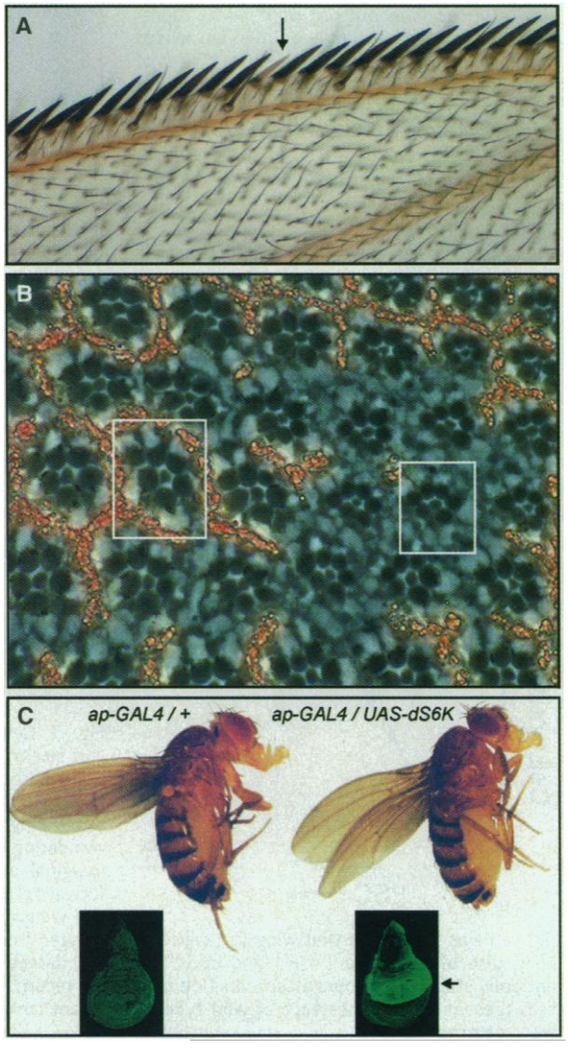

Fig. 4. Cell and compartment autonomous actions of dS6K. (**A**) Homozygous mutant sensory bristles at the wing margin and (**B**) homozygous mutant cells in the eye were induced by somatic recombination (*18*). (A) A y^- marked *dS6K* mutant sensory bristle is indicated by the arrow. (B) The w^+ wild-type and w^- marked homozygous *dS6K* mutant ommatidia are recognized by the presence (large box) or absence (small box) of red pigment, respectively. (**C**) Comparison of GAL4-*apterous* flies in the absence or presence of a transgene harboring a UAS response element coupled to an extra copy of *dS6K*. The insets show antibody staining of *dS6K* in imaginal wing discs, and the arrow indicates the dorsal-ventral compartment boundary (*20*, *21*). Posterior, right; dorsal, up.

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9. The gene was mapped to cytological position 64F1-3 as described (*26*). A collection of P elements in this region were analyzed for restriction fragment length polymorphisms, with one strain, *fs(3)07084*, showing an alteration when probed on Southern (DNA) blots with the *dS6K* cDNA.
10. Fly strains were as described [D. L. Lindsley and G. G. Zimm, *The Genome of* Drosophila melanogaster (Academic Press, San Diego, CA, 1992)] and were maintained under standard conditions. P-element revertants were generated by standard genetic techniques. *dS6K* and S6K cDNAs were from (*27*) and (*5*), respectively. To generate transgenic flies, we transformed a *y*, *w*, *ac* stock as described [G. M. Rubin and A. C. Spradling, *Science* **218**, 348 (1982)] with pBD1119 derivatives that contained either *dS6K*, S6K1, or S6K2 cDNAs under the control of the α-tubulin promoter [W. E. Theurkauf, H. Baum, P. C. Wensik, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8477 (1986)]. One copy of the *dS6K* or S6K transgene rescued the P-insertion or P-excision strains. The two additional *Minutes* analyzed were *M(3)66D* and *M(2)32A*, encoding ribosomal proteins L14 and S13, respectively (*7*).
11. Ovarian RNA was isolated and analyzed on Northern

(RNA) blots as described [M. J. Stewart and R. Denell, *Mol. Cell. Biol.* **13**, 2524 (1993)]. Plasmid rescue was as described [V. Pirrotta, in Drosophila, *a Practical Approach*, D. B. Roberts, Ed. (IRL Press, Oxford, UK, 1986), pp. 82–109]. Chimeric transcripts were reversed-transcribed with avian myeloblastosis virus (Promega) with an oligonucleotide priming from the exon 2 of the *dS6K* gene. PCR reaction products obtained revealed the presence of a chimeric mRNA containing the P-element sequence and the fusion of exon 1 to exon 2.

12. More severe alleles were obtained from the progeny of individual males carrying a Δ2-3 transposase and $dS6K^{07084}$ in trans with the deficiency Df(3L)64D-F [A. Garcia-Bellido, F. Cortes, M. Milan, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10222 (1994)], which did not complement the *dS6K* gene. Excision alleles were selected for their noncomplementation with the $dS6K^{07084}$ flies. To identify break points, we did PCR amplification from single flies [G. B. Gloor *et al.*, *Genetics* **135**, 81 (1993)], with oligonucleotides priming in the genomic sequence upstream of the P insertion point and in exon 8 of the *dS6K* gene.
13. The size of intervein cells was calculated by counting the number of hairs in a rectangle of 0.02 mm^2, located between veins 3 and 4 of the dorsal wing blade, up to the posterior cross vein. The total number of dorsal wing blade intervein cells was calculated by multiplying the number above by the area of the wing blade. For scanning electron microscopy, 3- to 4-day-old flies were anesthetized and immersed in 70% acetone. After critical point drying, they were mounted and coated with gold. The specimens were observed with a Hitachi S-800 field emission electron microscope at 6 kV.
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18. Somatic clones were generated with the FLP-FRT recombination system [T. Xu and G. M. Rubin, *Development* **117**, 1223 (1993)]. A 1-hour heat shock during the first larval instar permitted the recovery of clones in 50% of adults. Cross sections in eyes were performed as described [K. Basler, B. Christen, E. Hafen, *Cell* **64**, 1069 (1991)].
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22. When the wing blade is bent down such that it represents the arc of a quarter of a circle, the length of the dorsal wing sheet can be calculated from the equation $2L/\pi = (2L'/\pi) - d$, where L and L' represent the length of the ventral and dorsal wing sheets, respectively, and d is the distance between the middle of the two wing sheets. Because the length of the wing blade is 2 mm and the distance between the middle of the two wing sheets is about 10 μM [J. B. Tucker *et al.*, *Eur. J. Cell Biol.* **41**, 279 (1986)], the length of the dorsal sheet would measure 2.016 mm, a change of less than 1%.
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Affinity-Driven Peptide Selection of an NFAT Inhibitor More Selective Than Cyclosporin A

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The flow of information from calcium-mobilizing receptors to nuclear factor of activated T cells (NFAT)–dependent genes is critically dependent on interaction between the phosphatase calcineurin and the transcription factor NFAT. A high-affinity calcineurin-binding peptide was selected from combinatorial peptide libraries based on the calcineurin docking motif of NFAT. This peptide potently inhibited NFAT activation and NFAT-dependent expression of endogenous cytokine genes in T cells, without affecting the expression of other cytokines that require calcineurin but not NFAT. Substitution of the optimized peptide sequence into the natural calcineurin docking site increased the calcineurin responsiveness of NFAT. Compounds that interfere selectively with the calcineurin-NFAT interaction without affecting calcineurin phosphatase activity may be useful as therapeutic agents that are less toxic than current drugs.

Transcription factors of the NFAT family regulate immune responses as well as adaptive responses in heart and skeletal muscle (*1–3*). Four of the five NFAT proteins (NFAT1/p, NFAT2/c, NFAT3, and NFAT4/x) are cytoplasmic and are activated by stimulation of cell surface receptors coupled to Ca^{2+} mobilization (*1*). The Ca^{2+}-activated phosphatase calcineurin dephosphorylates these NFAT proteins, promoting their nuclear translocation and activation (*1*, *4*). Calcineurin docks at a site in the conserved NFAT regulatory domain that has the consensus sequence PxIxIT (*5*, *6*) (Fig. 1A). Interfering with docking of calcineurin at the PxIxIT sequence impairs NFAT activation and NFAT-dependent reporter gene expression (*5*).

To develop high-affinity NFAT inhibitors based on the PxIxIT sequence, we constructed combinatorial peptide libraries (*7*, *8*) (Fig. 1). The first library, with the sequence MA-xxxPxIxITxxHKK (where x represents a mixture of natural amino acid residues) was randomized in seven residues not fully conserved within the NFAT family (Fig. 1B). Peptides were selected for their ability to bind a glutathione S-transferase (GST) fusion protein containing the calcineurin catalytic domain (*8*). The peptide pool eluted from the calcineurin column showed moderate selection for glycine, serine, and lysine at position 3; no preferred residues at position 4; histidine or aliphatic residues at position 5; and moderate selection for polar residues (threonine, lysine, glutamine, and glutamic acid) at position 7 (Fig. 1B). Position 9 showed weak selection for aliphatic residues, notably valine. Glycine and proline were selected at positions 12 and 13, which suggests that the NFAT binding site in calcineurin imposes a turn at the COOH-terminal end of the PxIxIT motif.

To refine the peptide selection further, we

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