membranes (0.1 mg/ml) was determined as described [J. E. Kuster *et al.*, *J. Pharmacol. Exp. Ther.* **264**, 1352 (1993)]. Nonspecific binding was measured in the presence of 1 μM nonradioactive WIN-55212-2. FAAH activity was measured in rat brain particulate fractions as described (*13*). The uptake of [^{3}H]arachidonate (Amersham, 200 Ci/mmol; 5 nM brought to 100 nM) and [^{3}H]ethanolamine (Amersham, 50 Ci/mmol; 20 nM brought to 100 nM) was determined on cortical astrocytes for 4 min as described (*10*). The control uptake for [^{3}H]arachidonate was 16729 ± 817 dpm per well and for [^{3}H]ethanolamine it was 644 ± 100 dpm per well (n = 6).

18. Neurons or astrocytes were incubated for 4 min at 37°C in Krebs buffer containing [^{3}H]PGE_2 (0.67 nM brought to 100 nM with nonradioactive PGE_2; 171 Ci/mmol, New England Nuclear). After rinsing with Krebs buffer containing BSA, we subjected the cells to lipid extraction and counted radioactivity in the extracts. On average, neurons contained 245 ± 65 dpm per well and astrocytes 302 ± 20 dpm per well; nonspecific accumulation in astrocytes at 0° to 4°C was 355 ± 28 dpm per well (n = 6).
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26. The amounts of cAMP in the presence of a concentration of WIN-55212-2 below threshold (1 nM, determined in preliminary experiments) were 96.7 ± 2.5% of forskolin alone and were not significantly affected by 10 μM AM404 (89.8 ± 2.6%), 10 μM AM403 (92.4 ± 2.3%), or 10 μM bromcresol green (92.9 ± 2.3%) (n = 3). In the presence of a concentration of glutamate below threshold (3 μM) (*24*), cAMP concentrations were 91.6 ± 2% of forskolin alone and were not significantly affected by AM404 (84.4 ± 4.9%), AM403 (89.5 ± 2.4%), or bromcresol green (84.4 ± 3%) (n = 3).
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An NGF-TrkA–Mediated Retrograde Signal to Transcription Factor CREB in Sympathetic Neurons

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Nerve growth factor (NGF) is a neurotrophic factor secreted by cells that are the targets of innervation of sympathetic and some sensory neurons. However, the mechanism by which the NGF signal is propagated from the axon terminal to the cell body, which can be more than 1 meter away, to influence biochemical events critical for growth and survival of neurons has remained unclear. An NGF-mediated signal transmitted from the terminals and distal axons of cultured rat sympathetic neurons to their nuclei regulated phosphorylation of the transcription factor CREB (cyclic adenosine monophosphate response element–binding protein). Internalization of NGF and its receptor tyrosine kinase TrkA, and their transport to the cell body, were required for transmission of this signal. The tyrosine kinase activity of TrkA was required to maintain it in an autophosphorylated state upon its arrival in the cell body and for propagation of the signal to CREB within neuronal nuclei. Thus, an NGF-TrkA complex is a messenger that delivers the NGF signal from axon terminals to cell bodies of sympathetic neurons.

The growth and survival of many populations of neurons depends on trophic support provided by their target tissue (*1*). NGF is secreted by targets of sympathetic and some sensory neurons, and it is also expressed within discrete regions of the central nervous system (*1*, *2*). NGF belongs to a family of structurally related neurotrophic factors termed neurotrophins; this family includes brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (*2*). Two cell surface receptors for NGF have been identified: a receptor tyrosine kinase, TrkA, and the low-affinity neurotrophin receptor, $p75^{NTR}$. NGF exerts its growth- and survival-promoting effects on neurons through activation of TrkA and subsequent biochemical events that ultimately influence the expression of various genes, including those encoding ion channels, neurotransmitter-synthesizing enzymes, and cytoskeletal components (*3*).

NGF stimulates dimerization and autophosphorylation of TrkA and initiation of intracellular signaling cascades that propagate the signal to the nucleus (*4*). One transcription factor that is a key target of an NGF-stimulated signaling pathway is CREB (*5*). Upon exposure of pheochromocytoma-derived cell line PC12 to NGF, CREB becomes phosphorylated on its transcriptional regulatory site Ser^{133} (*5*), and this phosphorylation event promotes NGF activation of transcription of the immediate early gene *c-fos*. Because many NGF-regulated immediate early genes and delayed-response genes contain CREB binding sites within their upstream regulatory regions (*5*), CREB is likely to be a mediator of the general nuclear response to neurotrophins.

Because NGF is internalized and retrogradely transported from the axon terminal to the cell body (*6*), NGF itself may carry signals from the axon terminal to the nucleus. Alternatively, TrkA or $p75^{NTR}$, an NGF-receptor complex, or a terminally derived second messenger molecule might serve as a retrograde messenger (*7*). To address questions of retrograde NGF signaling, we used compartmentalized cultures of sympathetic neurons (*8*) and antibodies that distinguish between the Ser^{133}-phosphorylated and unphosphorylated states of CREB (anti–P-CREB) (*9*) and TrkA (anti–P-Trk) (Fig. 1A). In these cultures, the cell bodies are separated from the axon terminals and distal processes by a distance of either 1 mm or 3 to 4 mm, and the cell bodies and distal processes are located in separate fluid compartments (Fig. 1B). This system enables us to expose isolated terminals and distal axonal processes to NGF and then to assess by immunocytochemistry the phosphorylation state of CREB Ser^{133} and TrkA in cell bodies.

To determine whether NGF induces phosphorylation of CREB Ser^{133} in sympa-

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thetic neurons, we incubated neurons grown in compartmentalized cultures with medium containing a low concentration of NGF (2 ng/ml) for 48 hours. We then exposed either the cell bodies or distal axonal processes to medium containing a high concentration of NGF (200 ng/ml) for various times before fixation and anti–P-CREB immunocytochemistry (*10*). Exposure of either cell bodies or axon terminals and distal processes to NGF induced phosphorylation of CREB Ser^{133} within the nuclei of sympathetic neurons (Fig. 1C). Moreover, cell bodies and axon terminals were equally sensitive to NGF (Fig. 1D). However, the kinetics of this NGF-sensitive phosphorylation event differed depending on the site of NGF application or the distance between the cell bodies and the distal processes (Fig. 1E). Application of NGF directly to the cell bodies resulted in phosphorylation of CREB Ser^{133} within 5 min that returned to the basal level within 40 min. In contrast, upon application of NGF to terminals and distal processes of neurons whose cell bodies were located in center compartments 1 mm away, anti–P-CREB immunoreactivity peaked at 20 min and persisted for at least 1 hour. Furthermore, application of NGF to axon terminals and distal processes at least 3 mm away from the cell bodies resulted in appearance of nuclear P-CREB immunoreactivity that was first detected within 40 min (Fig. 1E). These results indicate that the messenger that transmits the NGF signal from distal axonal processes to CREB within the nucleus travels at a rate of approximately 2 to 4 mm/hour. ^{125}I-labeled NGF is retrogradely transported at an equivalent rate (*6*) or a slightly faster rate in sympathetic neurons (*11*).

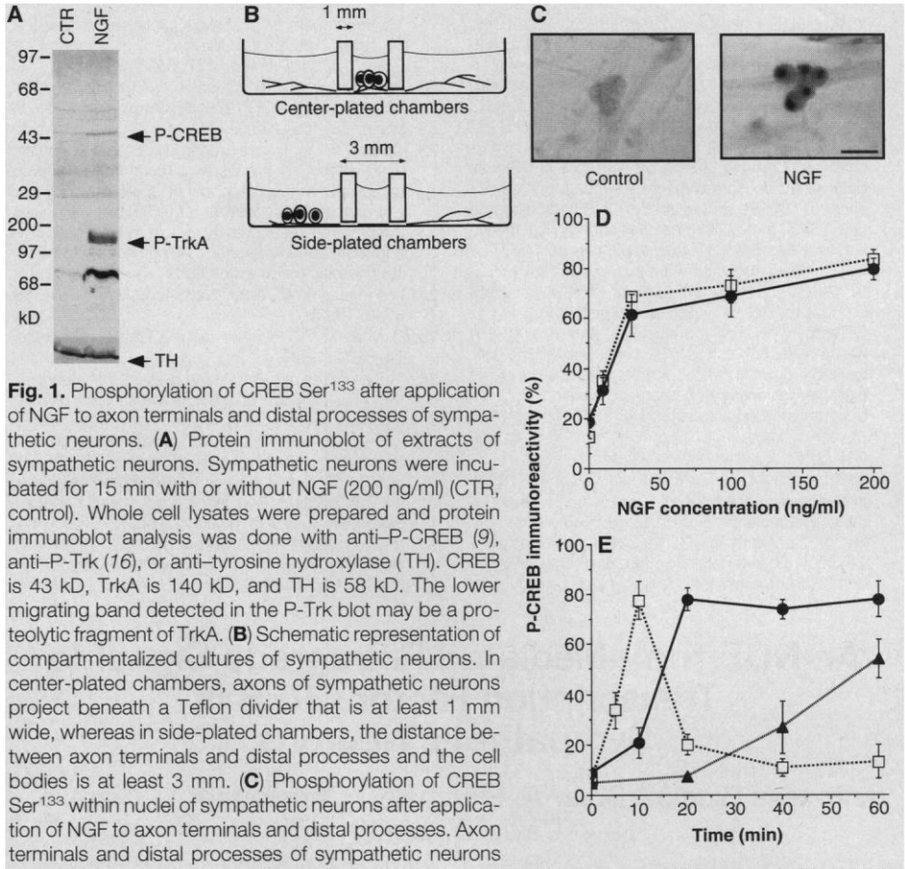

Fig. 1. Phosphorylation of CREB Ser^{133} after application of NGF to axon terminals and distal processes of sympathetic neurons. (**A**) Protein immunoblot of extracts of sympathetic neurons. Sympathetic neurons were incubated for 15 min with or without NGF (200 ng/ml) (CTR, control). Whole cell lysates were prepared and protein immunoblot analysis was done with anti–P-CREB (*9*), anti–P-Trk (*16*), or anti–tyrosine hydroxylase (TH). CREB is 43 kD, TrkA is 140 kD, and TH is 58 kD. The lower migrating band detected in the P-Trk blot may be a proteolytic fragment of TrkA. (**B**) Schematic representation of compartmentalized cultures of sympathetic neurons. In center-plated chambers, axons of sympathetic neurons project beneath a Teflon divider that is at least 1 mm wide, whereas in side-plated chambers, the distance between axon terminals and distal processes and the cell bodies is at least 3 mm. (**C**) Phosphorylation of CREB Ser^{133} within nuclei of sympathetic neurons after application of NGF to axon terminals and distal processes. Axon terminals and distal processes of sympathetic neurons grown in center-plated chambers were incubated in medium with or without NGF (200 ng/ml) for 20 min. Immunocytochemistry was done with anti–P-CREB, which recognizes CREB that is phosphorylated on Ser^{133} but not CREB that is unphosphorylated on this residue (*9*). The percentage of neurons that had nuclei stained with anti–P-CREB was determined by two individuals in blind analyses. Scale bar, 50 μm. (**D**) Dose-response analysis of NGF induction of CREB phosphorylation. Cell bodies (squares) or axon terminals and distal processes (circles) were treated with the indicated concentrations of NGF for 10 min (cell bodies) or 20 min (axon terminals and distal processes). Cells were then fixed and anti–P-CREB immunocytochemistry was performed. Values are means ± SEM of three independent experiments. (**E**) Kinetics of NGF induction of phosphorylation of CREB Ser^{133} in sympathetic neurons. Cell bodies of neurons grown in center-plated chambers (squares), terminals and distal axons of neurons grown in center-plated chambers (circles), or terminals and distal axons of neurons grown in side-plated chambers (triangles) were treated with NGF (200 ng/ml) for the indicated times, and then cells were fixed for immunocytochemistry with anti–P-CREB. Values are means ± SEM of three independent experiments performed in duplicate.

To determine whether internalization and retrograde transport of NGF are required for retrograde signaling to CREB within the nucleus, we prepared NGF that was covalently coupled to 1 μm–diameter microspheres (*12*). The NGF-coupled beads, but not control beads (*12*), induced autophosphorylation of TrkA (Fig. 2A) (*13*) and activation of the Ras-dependent protein kinase MAPK (mitogen-activated protein kinase) in PC12 cells and in sympathetic neurons (*14*). The NGF-coupled beads were not internalized by axon terminals and distal processes of sympathetic neurons, nor were they transported to cell bodies (*14*). Therefore, we used the NGF-coupled beads to determine whether activation of TrkA in terminals was sufficient for signaling to the cell body, or whether internalization and retrograde transport of NGF were also required.

Upon exposure of cell bodies of sympathetic neurons to either soluble NGF or NGF-coupled beads, phosphorylation of CREB Ser^{133} was detected in nuclei of nearly 80% of the neurons; this result was not seen with control beads (Fig. 2B). In contrast, NGF-coupled beads failed to stimulate CREB phosphorylation when applied to axon terminals and distal processes of sympathetic neurons (Fig. 2B). However, in parallel cultures, soluble NGF stimulated CREB phosphorylation in nearly 80% of neurons when applied to axon terminals. Thus, although internalization of NGF is not necessary for propagation of the NGF signal from the plasma membrane of the cell body to the nucleus, internalization and retrograde transport of NGF are critical for propagation of the NGF signal from the axon terminal and distal process to the nucleus. These results support a model in which NGF itself is a critical component of the retrograde signaling complex.

The possibility that NGF is retrogradely transported to the cell body of sympathetic neurons as part of a complex with one of its receptors, TrkA, is supported by the observation that NGF remains associated with tyrosine-phosphorylated TrkA in internalized vesicles purified from NGF-treated PC12 cells (*15*). We therefore tested whether exposure of terminals of sympathetic neurons to soluble NGF resulted in retrograde transport of autophosphorylated TrkA receptors. Appearance of tyrosine-phosphorylated TrkA (P-Trk) in cell bodies was determined by immunocytochemistry with antibodies that recognize TrkA only when it is phosphorylated on two tyrosine

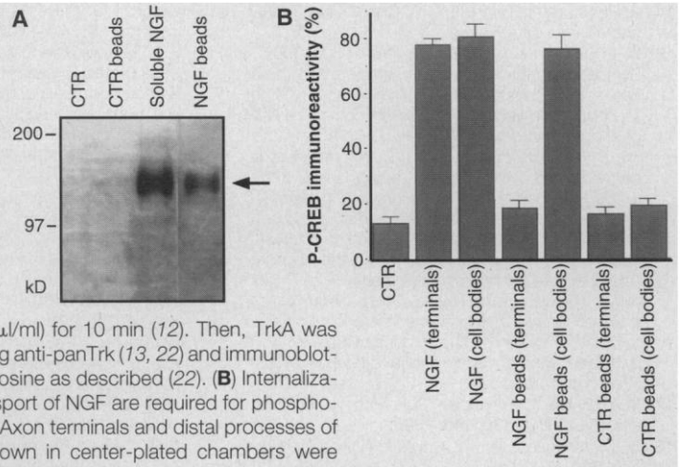

Fig. 2. Internalization and retrograde transport of NGF are critical for retrograde signaling to CREB. (**A**) Phosphorylation of TrkA induced by NGF-coupled beads, but not control (CTR) beads, in PC12 cells. PC12 cells were treated with control medium, control beads (4 μl/ml), NGF (100 ng/ml), or NGF-coupled beads (4 μl/ml) for 10 min (*12*). Then, TrkA was immunoprecipitated using anti-panTrk (*13, 22*) and immunoblotted with anti-phosphotyrosine as described (*22*). (**B**) Internalization and retrograde transport of NGF are required for phosphorylation of CREB Ser[133]. Axon terminals and distal processes of sympathetic neurons grown in center-plated chambers were treated with control medium, soluble NGF (200 ng/ml), NGF-coupled beads (4 μl/ml), or control beads for 20 min. Alternatively, cell bodies of neurons grown in center-plated chambers were treated with the same stimuli for 10 min. Cells were then fixed for immunocytochemistry with anti–P-CREB. Values are means ± SEM of three independent experiments performed in duplicate.

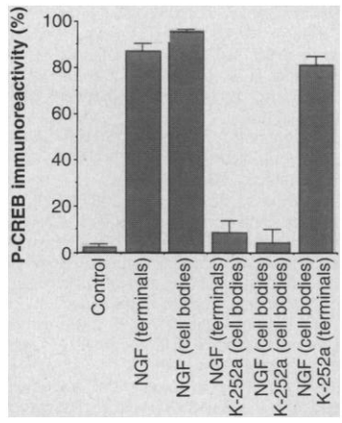

Fig. 4. Requirement of tyrosine kinase activity of retrogradely transported TrkA for propagation of the retrograde signal to CREB. Sympathetic neurons grown in center-plated chambers were untreated, or either cell bodies or axon terminals and distal processes were exposed to K-252a (100 nM, 30 min). Then, NGF was applied to axon terminals and distal processes or cell bodies, and P-CREB immunocytochemistry was done with anti–P-CREB. Values are means ± SEM of three independent experiments performed in duplicate.

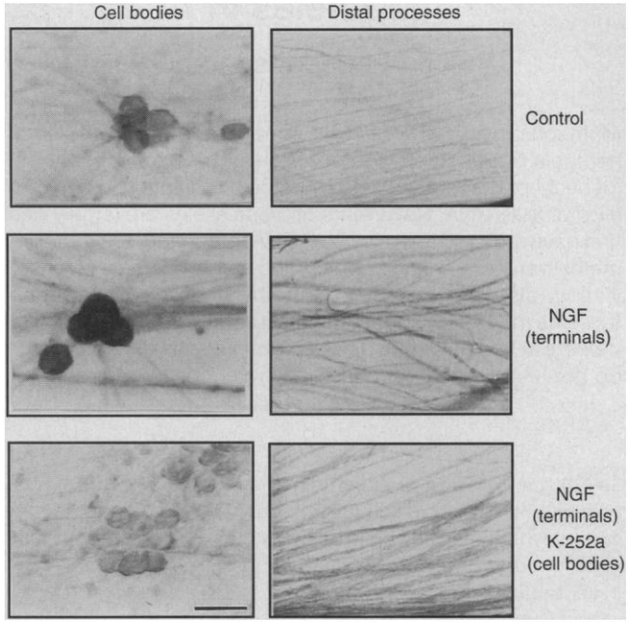

Fig. 3. Appearance of tyrosine-phosphorylated TrkA in distal processes and cell bodies after application of NGF to axon terminals and distal processes. Axon terminals and distal processes of neurons grown in center-plated chambers were untreated (control) or were treated with NGF (200 ng/ml) for 20 min. In the lower panels, the cell bodies were first treated with K-252a (100 nM, 30 min) and the terminals were then treated with NGF. Cells were fixed for immunocytochemistry with anti–P-Trk (Fig. 1A) (*16*). This experiment was performed three times with similar results. Incubation of anti–P-Trk with the phosphotyrosine-containing Trk peptide used to generate the antibody, but not control peptides, abolished P-Trk immunoreactivity (*14*). Scale bar, 50 μm.

residues, Tyr[674] and Tyr[675] (Fig. 1A) (*16*). Upon exposure of axon terminals and distal processes to NGF, the amount of P-Trk immunoreactivity was increased in the distal processes and also in cell bodies (Fig. 3), which suggests that P-Trk, like NGF, is retrogradely transported in sympathetic neurons. This conclusion is consistent with the observation that P-TrkA accumulates on the distal side of a ligation (*17*) or crush (*18*) of the sciatic nerve. Because NGF and TrkA remain associated within internalized vesicles (*15*), these results support a model in which NGF maintains cotransported TrkA in an active state.

To test this idea, we used a potent and selective inhibitor of Trk protein kinase activity, K-252a (*19, 20*). Application of K-252a to cell bodies prevented the appearance of P-Trk in cell bodies after exposure of axon terminals and distal processes to NGF (Fig. 3). In contrast, application of K-252a to cell bodies did not block NGF-induced accumulation of P-Trk immunoreactivity in distal axons and terminals. Thus, tyrosine kinase activity of retrogradely transported TrkA is critical for maintaining the receptor in an autophosphorylated state upon its arrival in the cell body.

We next tested the possibility that retrogradely transported, catalytically active TrkA contributes to retrograde signaling to the nucleus. For these experiments, we assessed the ability of terminally applied NGF to induce CREB phosphorylation in neurons in which we inhibited TrkA kinase activity in cell bodies but not in axon terminals and distal processes. When applied to cell bodies, K-252a blocked phosphorylation of CREB Ser[133] in response to application of NGF to axon terminals and distal processes (Fig. 4). In contrast, K-252a treatment of axon terminals and distal processes did not block phosphorylation of CREB Ser[133] in response to application of NGF directly to the cell bodies. We conclude that retrogradely transported, catalytically active TrkA and its ligand, NGF, are components of a complex that conveys the NGF signal from the axon terminals to CREB within the nuclei of sympathetic neurons.

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10. Sympathetic neurons were isolated from superior cervical ganglia of neonatal rats (*21*). Cells were plated in compartmentalized chambers (Tyler Research Products) as described (*8*) and grown in growth medium [Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), 5 μM arabinosylcytosine (Ara-C), and NGF (200 ng/ml)]. Medium was replaced every 3 days. After 4 to 7 days, medium within the chamber containing cell bodies was replaced with medium lacking NGF. This procedure resulted in the death of those neurons that had not extended processes into adjacent compartments. Neurons grown in center-plated chambers were used 2 to 3 weeks after plating. Neurons grown in side-plated chambers required a longer time to project through two barriers and were used 3 to 4 weeks after plating. For P-CREB immunocytochemistry experiments, medium was changed to contain a low concentration of NGF (2 ng/ml) for 48 hours before stimulation with NGF. Neurons were treated with NGF (200 ng/ml) and then fixed with acetone: methanol (1:1) for 3 min. Fixed cells were rinsed with phosphate-buffered saline (PBS) and permeabilized with PBS containing Triton X-100 (0.1%). After blocking with PBS solution containing horse serum (3%) and bovine serum albumin (BSA, 3%) at room temperature for 2 hours, cells were incubated with anti–P-CREB (1:1000 dilution) in the above solution at 4°C for 18 hours. Immune complexes were detected with an avidin biotin detection system (Vector Laboratories).
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13. TrkA was immunoprecipitated with anti-panTrk (*22*) from PC12 cells (7×10^6 cells per plate) treated with control medium, control beads (4 μl/ml), NGF (100 ng/ml), or NGF-coupled beads (4 μl/ml) (*13*), and phosphotyrosine protein immunoblotting was done as described (*22*). Similar results were obtained by immunoprecipitating TrkA from primary cultures of sympathetic neurons (*14*).
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16. Sympathetic neurons grown in center-plated chambers were incubated with medium without NGF for 12 hours, then treated with medium containing NGF (200 ng/ml) as described in Fig. 1. Cells were then fixed with PBS containing 4% paraformaldehyde, permeabilized with PBS containing Triton X-100 (0.1%), washed with PBS containing glycine (10 mM), and incubated in blocking solution [PBS containing BSA (3%) and normal goat serum (3%)] for 2 hours at room temperature. Cells were then incubated in blocking solution containing anti–P-Trk (1:250 dilution; New England Biolabs). This antibody specifically recognizes TrkA only when it is phosphorylated on Tyr^{674} and Tyr^{675}. The immune complexes were visualized with an avidin biotin detection system (Vector Laboratories).
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Hypermethylated *SUPERMAN* Epigenetic Alleles in *Arabidopsis*

Steven E. Jacobsen and Elliot M. Meyerowitz*

Mutations in the *SUPERMAN* gene affect flower development in *Arabidopsis*. Seven heritable but unstable *sup* epi-alleles (the *clark kent* alleles) are associated with nearly identical patterns of excess cytosine methylation within the *SUP* gene and a decreased level of *SUP* RNA. Revertants of these alleles are largely demethylated at the *SUP* locus and have restored levels of *SUP* RNA. A transgenic *Arabidopsis* line carrying an antisense methyltransferase gene, which shows an overall decrease in genomic cytosine methylation, also contains a hypermethylated *sup* allele. Thus, disruption of methylation systems may yield more complex outcomes than expected and can result in methylation defects at known genes. The *clark kent* alleles differ from the antisense line because they do not show a general decrease in genomic methylation.

DNA methylation is emerging as an important component of cell memory, the process by which dividing cells inherit states of gene activity. In mammals, methylation appears to play a key role in processes such as genomic imprinting and X-chromosome inactivation, and in plants methylation is correlated with a number of phenomena, including silencing of duplicated regions of the genome (*1*).

Arabidopsis mutants at the *DDM1* and *DDM2* loci have a reduced overall level of cytosine methylation and display a number of developmental defects (*2*). Transgenic *Arabidopsis* plants expressing an antisense cytosine methyltransferase RNA also exhibit abnormalities including a number of floral defects resembling the phenotypes of known floral homeotic mutants (*3*, *4*). These experiments suggest a direct cause and effect relation between DNA methylation and proper regulation of developmentally important genes. We describe here a class of epi-mutations in *Arabidopsis* that appear to be caused by overmethylation of the flower development gene *SUPERMAN* (*SUP*).

Seven independent mutants were identified [*clark kent* (*clk*) 1 through 7] with phenotypes similar to but weaker than that of the known *sup* mutants (*5*, *6*). Wild-type *Arabidopsis* flowers (Fig. 1A) contain six stamens (the male reproductive organs) and two central carpels that fuse to form the female reproductive structure. The *sup-5* allele (Fig. 1B) (*7*), which contains a nearly complete deletion of the *SUP* gene (*8*), produces an increased number of stamens [12.3 ± 0.3 (mean ± SE)] and carpels (2.9 ± 0.1) on the first 10 flowers produced on the plant. The *clk-3* allele (Fig. 1C) has an average of 7.8 ± 0.3 stamens and 3.4 ± 0.1 carpels, whereas the weaker *clk-1* allele has an average of 6.4 ± 0.1 stamens and

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