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8. The transcription factor construct AAV.CMVTF1 contains a CMV promoter (*15*) driving expression of a bicistronic gene with the following components: an activation domain fusion (the FRB fragment of human FRAP in which the threonine at amino acid 2098 was mutated to phenylalanine fused to an activation domain derived from the p65 subunit of human NF-κB), the internal ribosome entry site derived from encephalomyocarditis virus, the DNA-binding domain fusion (the ZFHD1 DNA-binding domain and three tandemly repeated copies of human FKBP12), and an 830–base pair (bp) portion of the rabbit β-globin gene containing the final intron and a 3′ untranslated region (UTR). The FRB-p65 and ZFHD1-3xFKBP fusion proteins both contain an NH_2-terminal epitope tag from influenza virus hemagglutin and nuclear localization signal from SV40 large T antigen (*4, 16*). Reporter construct AAV.Z12mEpo3S3 contains the 12 ZFHD1-binding sites (*4*), a minimal interleukin-2 promoter (*4*), a chimeric intron from pCI vector (Promega), a murine Epo cDNA, an SV40 late gene 3′ UTR, and a 2.7-kb stuffer containing a 1367-bp internal portion of human placental alkaline phosphatase coding sequence followed by a 626-bp portion of the human growth hormone 3′ UTR and a 720-bp portion of the 3′ rabbit β-globin intron and PAS (*4*). Reporter construct AAV.Z12rmEpo2S2 is similar except that the Epo cDNA is derived from a rhesus monkey Epo and the vector lacks the chimeric intron and the 720-bp rabbit β-globin portion of the stuffer. Reporter construct AAV.CMVrmEpo3 contains a human CMV enhancer-promoter, a chimeric intron from pCI vector, a rhesus monkey Epo cDNA, and an SV40 late gene 3′ UTR. Recombinant AAV was generated by cloning CMVTF1, Z12mEpo3S3, Z12rmEpo2S2, and CMVrmEpo3 expression cassettes into Xba I–restricted pSub 201 backbone (*17*). The resulting plasmids were used to produce recombinant AAV by a triple transfection method (*18*) or by an Ad/AAV hybrid system (*11*).
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12. An additional four rhesus monkeys were injected with a mixture of AAV vectors expressing Epo and the transcription factors as described in Fig. 4. Each responded to a dose of rapamycin (0.5 mg/kg) with a brisk and reversible induction of Epo with a concurrent increase in hematocrit. Group 1 had (transcription factor vector dose of 0.75×10^{13} genomes per kilogram and Epo vector dose of 1.0×10^{13} genomes per kilogram) peak plasma Epo levels of 220 and 420 mU/ml. Group 2 had (transcription factor vector dose of 0.75×10^{13} genomes per kilogram and Epo vector dose of 2.5×10^{13} genomes per kilogram) peak Epo levels of 560 and 120 mU/ml.
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19. Funding was provided by grants from the NIH [P30 DK47757-05 (J.M.W.) and P01 AR/NS43648-03 (H. L. Sweeney)], the Muscular Dystrophy Association, ARIAD Pharmaceuticals, and Genovo, a company J.M.W. founded and in which he holds equities. Technical support from N. Courage was greatly appreciated. W. Xiao provided plasmids for AAV production.

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Processing of the Notch Ligand Delta by the Metalloprotease Kuzbanian

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Signaling by the Notch surface receptor controls cell fate determination in a broad spectrum of tissues. This signaling is triggered by the interaction of the Notch protein with what, so far, have been thought to be transmembrane ligands expressed on adjacent cells. Here biochemical and genetic analyses show that the ligand Delta is cleaved on the surface, releasing an extracellular fragment capable of binding to Notch and acting as an agonist of Notch activity. The ADAM disintegrin metalloprotease Kuzbanian is required for this processing event. These observations raise the possibility that Notch signaling in vivo is modulated by soluble forms of the Notch ligands.

The Notch (N) signaling pathway defines an evolutionarily conserved cell interaction mechanism that controls cell fate by modulating the cell's response to developmental signals (*1, 2*). The N receptor is cleaved in the trans-Golgi network as it traffics toward the plasma membrane and eventually forms a ligand-competent heterodimeric molecule (*3*). Both known ligands, Delta (Dl) and Serrate (Ser), are thought to act as transmembrane proteins that interact via their extracellular domains with N receptors that are expressed on adjacent cells (*2, 4*). Given the similar phenotypes produced by loss of Notch signaling and loss-of-function mutations in the *kuzbanian* (*kuz*) gene [a gene encoding a putative member of the ADAM family of metalloproteases (*5*)], it has been suggested that Kuz may be involved in the cleavage of N (*6*). This hypothesis is not corroborated by recent biochemical studies, indicating that the functionally crucial cleavage of N in the trans-Golgi network is catalyzed by a furinlike convertase (*7*).

A genetic screen to identify modifiers of the phenotypes associated with the constitutive expression of a dominant negative transgene of *kuz* (*kuzDN*) in developing imaginal discs identified *Delta* as an interacting gene (*8*). Flies expressing this dominant negative *kuz* construct, despite carrying a wild-type complement of *kuz*, became semi-lethal when heterozygous for a loss-of-function *Delta* mutation (*8*). In contrast, *Delta* duplications rescued the phenotypes associated with *kuzDN* (Fig. 1). The *kuzDN* flies display extra vein material (especially deltas at the ends of the longitudinal veins), wing notching (observed with a low penetrance), extra bristles on the notum, and have small rough eyes (Fig. 1, A and E) (*6*). When *kuzDN* flies carried three, as opposed to the normal two, copies of wild-type *Notch*, the bristle and eye phenotypes were not affected (*8*), nor were the vein deltas altered (Fig. 1D). However, the *kuzDN* phenotypes were effectively suppressed by *Delta* duplications (Fig. 1, B and F), indicating that a higher copy number of Dl molecules is capable of overriding the effect of the *kuzDN* construct.

The interaction between *Delta* and *kuz* was further explored through their respective protein products. Dl antigen was expressed in a stably transfected S2 cell line and was examined with an extracellular domain–specific antibody (*9*) (Fig. 2A). A fragment migrating

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faster than Dl was observed exclusively in the medium. The size of this fragment, about 67 kD (Fig. 2C), is consistent with the extracellular domain of Dl, estimated to be 65 kD (Fig. 2D). This fragment was subsequently affinity-purified from the culture medium, and the NH_2-terminal sequence was determined (Fig. 2E). The sequence revealed a putative propeptide processing site that is conserved in all the Delta homologs (Fig. 2E). Thus, Dl may be cleaved at the cell surface to release a soluble fragment, designated as Dl^{EC} (Delta extracellular domain). Protein immunoblot analysis of *Drosophila* embryos revealed the existence of both Dl and a fragment with the same mobility as Dl^{EC}, which implies that the same Dl-derived product is present in vivo (Fig. 2B). Between Dl and Dl^{EC}, additional potentially transient proteolytic products were detectable (Figs. 2B and 3D; $kuz^{+/-}$).

The possibility that the generation of Dl^{EC} can be influenced by Kuz was examined by cotransfection experiments in S2 cells that express wild-type Kuz endogenously (*6*). Cotransfection of Dl with Kuz showed an increase in the Dl^{EC} fragment as compared to Dl transfection alone (Fig. 3A). The corresponding decrease in Dl suggests that Dl is the precursor of the Dl^{EC} product. These data also indicate that transfection of Kuz acts additively to the endogenous Kuz in the S2 cells. Supporting this hypothesis, cotransfection with KuzDN had an inhibitory effect on Dl^{EC} production (Fig. 3A). Under identical experimental conditions, cotransfection of Kuz or KuzDN had no effect on the proteolytic processing of N (Fig. 3B). Thus, Kuz functions in the processing of Dl but not of N. In agreement with this conclusion, Dl^{EC} production was markedly inhibited by the metalloprotease inhibitors EDTA and 1,10-phenanthroline (Fig. 3C), whereas no effect was observed with serine protease inhibitors (phenylmethylsulfonyl fluoride and aprotinin), cysteine protease inhibitor (leupeptin), or aspartyl protease inhibitor (pepstatin) (*10*).

The role of Kuz in generating this product in vivo was examined in *kuz* mutants. *kuz* maternal null embryos with either one ($kuz^{+/-}$) or no ($kuz^{-/-}$) zygotic copies of *kuz* were created by crossing female flies carrying *kuz* germline clones (*5*). The $kuz^{-/-}$ embryos were distinguished from $kuz^{+/-}$ embryos by the absence of malpighian tubules and the lack of movement in the $kuz^{-/-}$ embryos. Extracts prepared from a collection of nine of each type of embryo

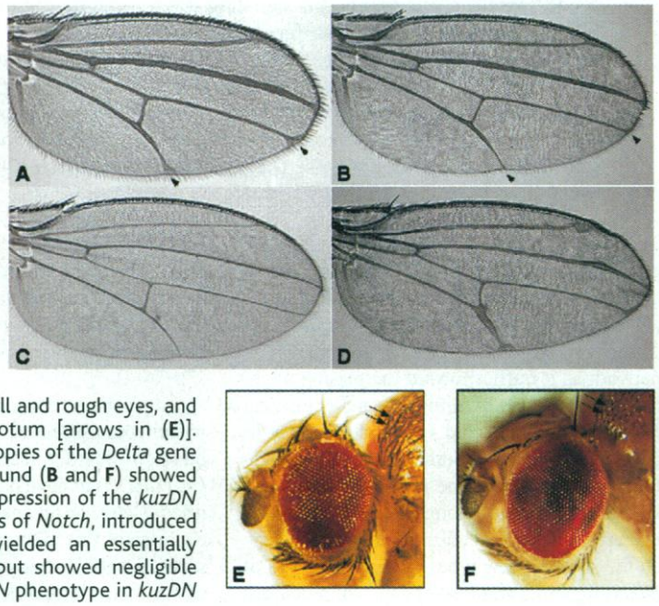

Fig. 1. Modifiers of the *kuz* phenotype. A genetic modifier screen was carried out to identify genes that interact with *kuz*. In the screen, a strain was used that constitutively expresses a *kuzDN* construct (*18*) in developing imaginal discs. Adult mutant phenotypes (*19*) of these flies included extra wing vein elements, mostly notably deltas at the ends of the longitudinal veins [arrowheads in (**A**)], small and rough eyes, and extra bristles on the notum [arrows in (**E**)]. Flies that carried three copies of the *Delta* gene with the *kuzDN* background (**B** and **F**) showed an almost complete suppression of the *kuzDN* phenotypes. Three copies of *Notch*, introduced by a transgene (*20*), yielded an essentially normal phenotype (**C**) but showed negligible suppression of the *kuzDN* phenotype in *kuzDN* flies (D).

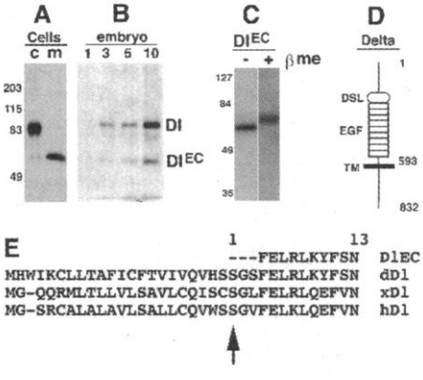

Fig. 2. A soluble Delta fragment is released constitutively in Delta-S2 cell culture and in vivo. (**A**) Expression of Dl antigen in stably transfected S2 cells (*17*) is detected by SDS-PAGE and protein immunoblotting with monoclonal antibody 9B (*9*) in nonreduced cell extracts (c) and culture medium (m). A product consistent with Dl is detected in the cell extract. A product of greater mobility is seen in the medium that is consistent in size with the extracellular domain of Dl and is referred to as Dl^{EC}. (**B**) Bands of the same mobility are seen in extracts of 16-hour wild-type *Drosophila* embryos. The number of embryos loaded on the gel is shown above the lanes. (**C**) Affinity-purified Dl^{EC} (*21*) migrates with a molecular mass of about 62 and 67 kD in nonreducing (lane 1) and reducing (lane 2) conditions, respectively, on Coomassie blue–stained SDS-PAGE. (**D**) A schematic of *Drosophila* Dl illustrates the conserved Delta Serrate, Lag-2 domain (DSL), the epidermal growth factor (EGF)–like repeats, and the transmembrane domain (TM). Amino acid numbering of the NH_2-terminus, the beginning of the TM domain, and the COOH-terminus are shown. (**E**) Thirteen cycles of NH_2-terminal amino acid sequence analysis of Dl^{EC} (DlEC) are shown with alignment to the sequences of *Drosophila* (dDl), *Xenopus* (xDl), and human (hDl) Delta proteins. The arrow indicates the conserved serine in the position of the NH_2-terminus of Dl^{EC} and the putative signal peptide processing site for Dl.

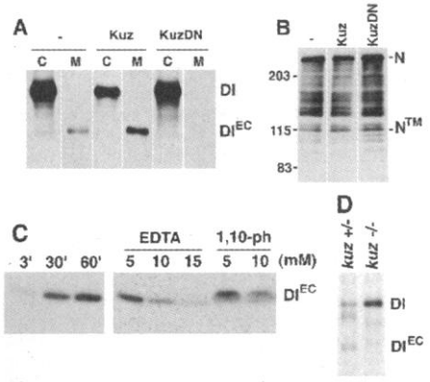

Fig. 3. Kuz plays a direct role in Delta processing in vitro and in vivo. (**A**) Dl and Dl^{EC} were visualized by protein immunoblotting with the 9B antibody in the cell pellet (c) and the medium (m) in S2 cells transiently transfected with Dl alone [pMTDl (*22*) lanes 1 and 2], cotransfected with Kuz (*6*, *23*) (lanes 3 and 4) or cotransfected with Kuz DN (*6*, *23*) (lanes 5 and 6). (**B**) Cotransfection of Kuz and KuzDN with N [pMTNMg (*22*)], done under identical experimental conditions as for Dl and protein immunoblotted with an intracellular domain–specific antibody (*22*), yielded a negligible effect on the processing of N as seen by the invariant levels of N^{TM}, the constitutively processed form of N (*3*). (**C**) The metalloprotease inhibitors EDTA and 1,10-phenanthroline (1,10-ph) inhibited the endogenous S2 cell proteolytic activity that produced Dl^{EC}. The left panel shows the accumulation of Dl^{EC} at various time points up to 60 min in the medium of S2 cells stably expressing Dl. The right panel shows the accumulation of Dl^{EC} at 60 minutes in the presence of EDTA and 1,10-phenanthroline. Both of these reagents, which are well-documented metalloprotease inhibitors, inhibited accumulation of Dl^{EC} in the medium. (**D**) Dl processing was inhibited in $kuz^{-/-}$ embryos. Nine $kuz^{+/-}$ and $kuz^{-/-}$ embryos were identified by morphology, and the extracts were analyzed by SDS-PAGE and protein immunoblotting with 9B. Dl^{EC} was absent in $kuz^{-/-}$ embryos and demonstrated a higher level of Dl as compared to $kuz^{+/-}$ embryos.

showed the distinct absence of Dl^{EC} and higher levels of Dl in the $kuz^{-/-}$ embryos as compared to $kuz^{+/-}$ embryos (Fig. 3D). Reprobing of the same membrane with antibody to N showed no difference in the processing of N in the $kuz^{-/-}$ and $kuz^{+/-}$ embryos (*10*). Furthermore, analysis of 14 randomly selected individual embryos showed 8 embryos with high levels of Dl (*10*), analogous to the $kuz^{-/-}$ embryos (Fig. 3D) and consistent with the predicted numerical outcome of the cross. Together, these observations indicate that Kuz mediates the proteolytic processing of Dl in vivo.

Although *kuz* mutants have multiple defects, indicating an involvement in several different processes (*5*), their phenotypes partially overlap with that of *Delta* mutants. Inactivation of *kuz* during embryogenesis causes a more extensive neurogenic phenotype than do *Delta* mutations; nevertheless, it is clear that in the ventrolateral region the neural hypertrophy in the two mutations is identical. Similarly, due to the pleitropy of *kuz* and *Delta* the phenotypes associated with mosaic clones are complex. Yet they are also partially overlapping, compatible with the hypothesis that the processing of the Dl protein is mediated by Kuz (*5, 6, 11*).

Dl^{EC} bound specifically to N-expressing S2 cells (Fig. 4A), suggesting that a Dl^{EC}-N complex forms on these cells. These results were extended by analysis of the ability of Dl^{EC} to compete for Dl binding to N in a cell aggregation assay (Fig. 4B). Preincubation of the N cells with Dl^{EC} concentrate (*16*) resulted in a reduction in the initial rate of aggregation with Dl cells. The competitive effect of Dl^{EC} was sensitive to the concentration added and to the time of preincubation with the N cells (*10*). Furthermore, preincubation of the Dl cells with Dl^{EC} had no effect on subsequent aggregation with N cells, indicating that Dl^{EC} specifically binds to N in a competitive manner with respect to Dl.

The biological activity of Dl^{EC} was examined in a cell culture assay. Neurons develop axodendritic processes (such as neurites) in primary cultures of mouse embryonic cerebrum (Fig. 4C). Sestan *et al.* (*12*) have demonstrated that ligand-dependent Notch activation in cortical neurons, which express endogenous Notch receptors, causes morphological changes as well as retraction of neurites. The same effects were observed when the neurons were cultured in the presence of enriched Dl^{EC}-containing medium or purified Dl^{EC} (Fig. 4C). Thus, Dl^{EC} has biological activity and apparently acts as an agonist of Notch activity.

Genetic and biochemical evidence demonstrate that proteolytic processing of Dl produces the soluble Dl^{EC} fragment, which is biologically active with an apparent agonistic function in the Notch pathway. Previous studies involving in vivo expression of artificially truncated Notch ligands, in *Drosophila* and other systems, have demonstrated both agonistic and antagonistic activities (*13, 14*). A soluble form of Delta (DIS) can act as an antagonist in the developing *Drosophila* eye (*13*). However, Dl^{EC} is not identical to DIS, and therefore it is plausible that the two molecules may be functionally different.

Although Kuz does not appear to be responsible for the constitutive cleavage of N, the possibility that Kuz can cleave N at alternative sites remains. In this regard, it has been claimed that KuzDN is able to inhibit transactivation of a target gene of the N pathway induced by ligand binding to the receptor (*7*). However, it is possible that this effect does not reflect N cleavage but rather the cleavage of Dl to produce an active ligand. Kleug *et al.* (*15*) have recently reported the processing of Dl during normal embryogenesis, demonstrating the existence of Delta fragments, one of which is consistent with Dl^{EC}. The intermediate forms detected in embryos 16 to 20 hours old (Figs. 2B and 3D; $kuz^{+/-}$) were not present in *kuz* mutants (Fig. 3D; $kuz^{-/-}$), raising the possibility that the generation of these products may also be mediated by Kuz. The importance of additional cleavages in Dl, the mode of activity of full-length Dl, and whether the second ligand Ser is also processed are critical questions to resolve. It is now apparent that future analyses of Delta in Notch signaling events must consider its potential as a diffusable ligand.

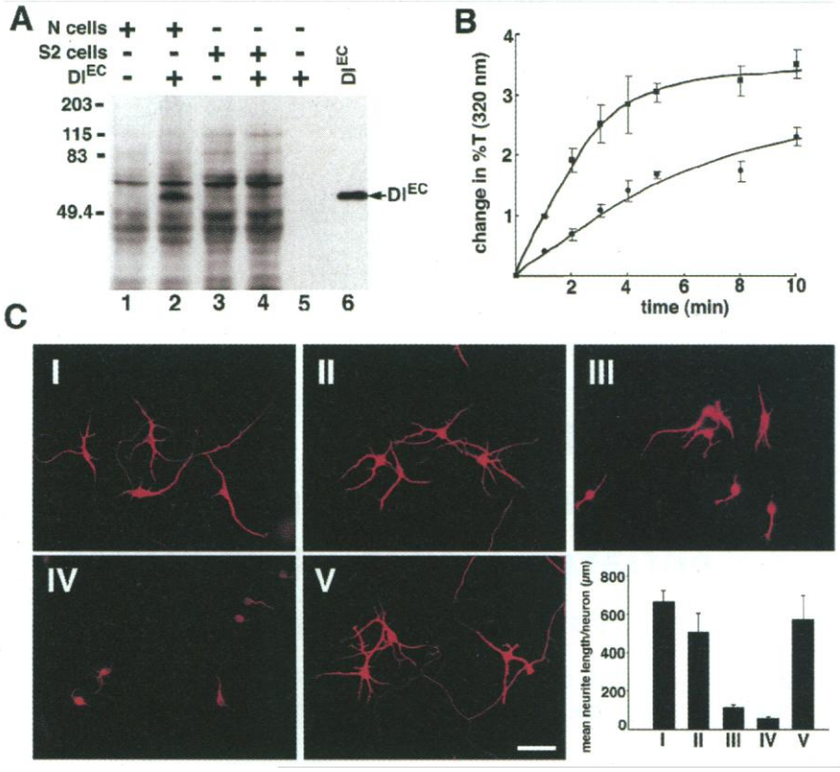

Fig. 4. Dl^{EC} binds to Notch, competes for Notch-Delta interaction, and acts as an agonist. (**A**) The Dl^{EC} fragment specifically binds to N-expressing S2 (N-S2) cells and does not bind to S2 cells alone. N-S2 cells (lanes 1 and 2), incubated in the absence (lane 1) or presence (lane 2) of Dl^{EC} (lane 6), were sedimented through a sucrose cushion, and the extract was protein immunoblotted with antibody 9B (*16*). Lanes 3 and 4 show parallel incubations with S2 cells in the absence (lane 3) or presence (lane 4) of Dl^{EC}. Lane 5 shows Dl^{EC} sedimented in the absence of cells. (**B**) Preincubation of N-S2 cells with Dl^{EC} concentrate reduced the subsequent rate of aggregation with Dl-S2 cells as measured turbidimetrically with transmitted light at 320 nm (*24*). At the concentration shown [1× Dl^{EC}, solid circles, (*24*)] a 60% inhibition in the initial rate of aggregation was seen as compared to control medium concentrate [1× ΔECN, solid squares, (*24*)]. The error bars show the standard deviation of the mean of triplicate determinations. (**C**) The effect of Dl^{EC} on primary cultured cortical neurons 7 to 10 days old (*12*) is shown in the representative images as follows: (I) before treatment, (II) cultured in the presence of ΔECN medium, (III) cultured in the presence of Dl^{EC} medium, (IV) cultured in the presence of affinity-purified Dl^{EC}, and (V) buffer control for purified Dl^{EC}. The graph represents the mean length of neurites per neuron. Each bar represents the mean ± SEM of three separate experimental trials. Primary cortical neurons exhibited multipolar morphology and the extensive neurite network in control cultures (I), in cultures in the presence of ΔECN medium (II), and in a buffer control for purified Dl^{EC}. A significant decrease in the mean neurite length per neuron and limited neurite branching in cultures treated with Dl^{EC} medium (III) and purified Dl^{EC} (IV) is seen. Scale bar, 50 μm.

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18. Expression of a *KuzDN* construct lacking the proprotein and metalloprotease domains was driven by a GAL4 line 32B.
19. More than 2400 lethal P-element insertions were screened for phenotypic modification effects on *KuzDN*. Seven P-element insertions reduced viability of the *KuzDN* flies (were semi-lethal) when the flies were also heterozygous for each of the P insertions. Preliminary characterization of these P insertions revealed that two of them were *kuz* alleles and one was a loss-of-function *delta* allele; the nature of the other insertions is unknown.
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21. The IgG fraction of 9B ascites fluid was purified with a HiTrap recombinant protein A–Sepharose (Pharmacia) and subsequently coupled to CNBr-activated Sepharose (Pharmacia). *Drosophila* Dl-S2 cells were induced with 0.7 mM $CuSO_4$ in serum-free medium for 2 days, and the medium was collected and precipitated with 70% ammonium sulfate. The precipitate was collected by centrifugation and subsequently resuspended and dialyzed against 20 mM Hepes, 150 mM NaCl, and 2 mM $CaCl_2$ (pH 7.4). This sample was passed over the 9B-Sepharose, washed with 1.0 M NaCl, and eluted with 25 mM glycine, pH 2.8, and immediately neutralized with 1M tris-HCl. NH_2-terminal amino acid analysis was performed with an Applied Biosystems gas phase sequencer in the Keck Foundation Biotechnology Resource Laboratory, Yale University.
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23. The *KuzDN* construct used for the genetic screen and full-length *kuz* were cloned into pCaSpeR3 for expression under the heat shock promoter. Cotransfections with these constructs and those of Pan *et al.* (*6*) (Fig. 3, A and B) gave identical results.
24. Expression of N and Dl in S2 cells was induced for 16 hours with 0.085 mM and 0.022 mM $CuSO_4$, respectively. The cells were then centrifuged and raised in serum-free medium to an equivalent density yielding between 20 and 30% transmitted light at 320 nm (T_{320nm}) (~2×10^6 cells per milliliter) in a Benchtop spectrophotometer. Blank values were set with M3 medium alone. Four hundred microliters of N cells and 400 μl of Dl cells were then pipetted into a 1.4-ml, black-sided, stoppered quartz cuvette that was then quickly inverted three times. The T_{320nm} was read immediately to determine the time "zero" value. The cuvette was then rocked horizontally on a Thermolyne vari-mixer at 20 oscillations per minute, and subsequent T_{320nm} readings were taken at 1-min intervals. Changes in T_{320nm} (relative to time zero) were then plotted versus time. The effect of Dl^{EC} was compared to a control concentrate of medium from S2 cells stably transfected with an irrelevant construct [ΔECN (*12*)]. N-S2 cells (400 μl) were preincubated with either 5× Dl^{EC} or 5× ΔECN (100 μl) for 10 to 60 min and subsequently aggregated with Dl-S2 cells as described above.

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Plant Paralog to Viral Movement Protein That Potentiates Transport of mRNA into the Phloem

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CmPP16 from *Cucurbita maxima* was cloned and the protein was shown to possess properties similar to those of viral movement proteins. *CmPP16* messenger RNA (mRNA) is present in phloem tissue, whereas protein appears confined to sieve elements (SE). Microinjection and grafting studies revealed that CmPP16 moves from cell to cell, mediates the transport of sense and antisense RNA, and moves together with its mRNA into the SE of scion tissue. CmPP16 possesses the characteristics that are likely required to mediate RNA delivery into the long-distance translocation stream. Thus, RNA may move within the phloem as a component of a plant information superhighway.

Phloem represents an advanced long-distance transport system that delivers nutrients and hormones to plant tissues and organs. Mature SE are enucleate (*1*) and thus must rely on their associated companion cells (CC) for maintenance of their physiological functions (*2*). To this end, SE are connected to CC through specialized, branched plasmodesmata (*3*) that mediate delivery of proteins into the long-distance translocation stream (*4, 5*).

The observation that specific mRNA molecules, such as sucrose transporter 1 (*SUT1*)