

A translational block to HSPG synthesis permits BMP signaling in the early *Drosophila* embryo

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Heparan sulfate proteoglycans (HSPGs) are extracellular macromolecules found on virtually every cell type in eumetazoans. HSPGs are composed of a core protein covalently linked to glycosaminoglycan (GAG) sugar chains that bind and modulate the signaling efficiency of many ligands, including Hedgehog (Hh), Wingless (Wg) and Bone morphogenetic proteins (BMPs). Here, we show that, in *Drosophila*, loss of HSPGs differentially affects embryonic Hh, Wg and BMP signaling. We find that a stage-specific block to GAG synthesis prevents HSPG expression during establishment of the BMP activity gradient that is crucial for dorsal embryonic patterning. Subsequently, GAG synthesis is initiated coincident with the onset of Hh and Wg signaling which require HSPGs. This temporal regulation is achieved by the translational control of HSPG synthetic enzymes through internal ribosome entry sites (IRESs). IRES-like features are conserved in GAG enzyme transcripts from diverse organisms, suggesting that this represents a novel evolutionarily conserved mechanism for regulating GAG synthesis and modulating growth factor activity.

KEY WORDS: Heparan sulfate proteoglycans, HSPG, Glycosaminoglycan, GAG, Bone morphogenetic protein signaling, *Drosophila* embryonic patterning, Decapentaplegic, Dpp, Hedgehog, Wingless

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are widely expressed glycoproteins that are crucial for embryonic development, as well as for adult physiology and homeostasis. HSPGs consist of glycosaminoglycan (GAG)-anchored, membrane-spanning, or secreted, core proteins, which are modified by the addition of linear polysaccharide chains built from repeating glucuronic acid/N-acetylglucosamine (GAG) disaccharide units. HSPG GAG chains undergo regulated processing through deacetylation, N-sulfation/desulfation and epimerization to generate diverse structures that mediate interactions with a large number of ligands, including the axon guidance cue Slit, Hedgehog (Hh), Wingless (Wg/Wnt), Fibroblast growth factor (FGF), Transforming growth factor β (TGF β) and BMPs. HSPG binding modulates growth factor signaling through several distinct mechanisms (Bishop et al., 2007; Esko and Selleck, 2002; Hacker et al., 2005; Kreuger et al., 2006; Lin, 2004). For example HSPGs have been shown to affect the distribution of ligands such as Hh, Wg and Decapentaplegic (Dpp), the *Drosophila* ortholog of BMP2/BMP4, by affecting their stability, localization and/or transport (Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004; The et al., 1999). In FGF signaling, HSPGs act as co-receptors that promote ternary complex formation by binding to both ligand and receptor. HSPGs are also believed to modulate signaling by acting as low-affinity reservoirs for growth factors, thereby altering the effective ligand concentration at the cell surface (Eswarakumar et al., 2005). Additionally, HSPGs are likely to affect growth factor signaling indirectly through their interactions with lipases, proteases, protease inhibitors and extracellular matrix proteins. Given the ability of HSPGs to regulate several growth factor pathways at multiple levels, determining how their activity is spatially and temporally regulated is crucial to understanding HSPG

specificity for different ligands and their role in development and disease. As HSPGs are thought to be present on all adherent cells (Bishop et al., 2007), it is believed that specificity is generated by spatially and temporally regulated modifications of the disaccharide chains, rather than from regulation of their synthesis (Bulow and Hobert, 2006).

In *Drosophila*, Hh, Wg and BMP growth factors play cardinal roles in patterning and cell fate specification in the embryo and in larval imaginal discs. In the wing disc, Hh is expressed in the posterior compartment, and signals at short range to induce Dpp expression in an adjacent anterior stripe of cells. Localized Dpp expression results in the generation of a concentration gradient centered at the anterior/posterior compartment boundary that specifies cell fate across the wing pouch. Wg is expressed in a narrow stripe perpendicular to Hh and Dpp, and regulates target gene expression along the dorsoventral (DV) axis. Clonal analysis in the wing disc has shown that mutations in genes for several GAG synthetic enzymes, including *tout-velu* (*ttv*) and *sister of tout-velu* (*sotv*; *Ext2* – FlyBase), which encode GAG polymerase subunits, and *brother of tout-velu* (*botv*), an N-acetylglucosamine Transferase-I/II required for the initiation of heparan synthesis, result in strongly reduced levels of extracellular Hh, Wg and Dpp, indicating that HSPGs are required for ligand distribution (Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004). Furthermore, expression of Hh, Wg and Dpp target genes is compromised in clones of cells where GAG synthesis is disrupted, demonstrating that HSPG function is crucial for signaling by these growth factors (Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004; The et al., 1999).

Previous studies have established that embryos lacking HSPG activity are defective in Hh and Wg signaling (reviewed by Hacker et al., 2005). However, although it is often assumed that HSPGs participate in shaping the Dpp gradient (Kerszberg and Wolpert, 2007), their role in embryonic BMP signaling has not been extensively examined. Here, we show that HSPGs play no role in BMP signaling in the early embryo, and, in fact, are absent during the first three hours of embryonic development when the BMP gradient is established. HSPGs are not expressed despite maternal loading of transcripts for all known HSPG biosynthetic enzymes.

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We demonstrate that the tight temporal regulation of HSPG biosynthesis is achieved through a translational control mechanism based on internal ribosome entry. Transcripts for GAG biosynthetic enzymes from other species share features indicative of translational control (Grobe and Esko, 2002), suggesting that this may represent a novel and conserved strategy for the temporal and spatial regulation of HSPG activity and growth factor signaling.

MATERIALS AND METHODS

Genetics

UAS-ttv-myc (The et al., 1999), UAS-dlp and UAS-dally-myc were generously provided by Drs Norbert Perrimon (Harvard) and Hiroshi Nakato (University of Minnesota). The strong maternal α tubulin67c>Gal4 (matTub>Gal4) driver that directs expression of UAS transgenes in the ovarian germline was obtained from Antoine Guichet (Institut Jacques Monod, Paris). Other stocks were obtained from the Bloomington Stock Center. For depletion experiments, *ttv*^{k11904}, *Hsp70*>Gal4/CyO; UAS-Ttv/UAS-Ttv flies were mated to *ttv*⁰⁰⁶⁸¹, *sotv*¹⁸¹/CyO; Tub>Gal80^{ts}/Tub>Gal80^{ts} flies. Progeny collected at room temperature for 3 days were transferred to 30°C to inactivate the Gal80^{ts} repressor and heat shocked daily to induce UAS-Ttv expression. Rescued *ttv* homozygous females were mated with *ttv*²⁰⁵⁵/CyO males and maintained at room temperature to enable Gal80^{ts} to block UAS-Ttv expression.

Perivitelline injection

Freshly laid embryos were dechorionated, covered with Halocarbon oil and, at 1 to 1.5 hours of development, 30 μ l of Heparin/PBS (Sigma) was injected into the posterior perivitelline space using an IM300 programmable microinjector (Narishige). Heparin solutions were at 0.1, 1 and 10 mg/ml resulting in final concentrations in the perivitelline fluid (PVF) of 0.15, 1.5 and 10.5 μ g/ml based on a PVF volume of 20 nl. Inhibition of Dpp signaling in wild-type embryos was observed at PVF concentrations of 1.5 μ g/ml. A tenfold higher heparin concentration, 10.5 μ g/ml, was required for the inhibition of *Kr-lacZ* expression in *dl*⁻ embryos compared with in wild type. This may reflect the fact that *dpp* is expressed ubiquitously in this genotype. Embryos were incubated for 21 hours at 18°C under high humidity, fixed in glutaraldehyde/heptane, hand devitellinized and stained for β -galactosidase activity before mounting.

Western blots

For Dally, Dlp and GFP westerns, UAS transgenic lines were crossed to flies homozygous for the strong maternal driver α tubulin67c>GAL4. For staged samples, eggs/embryos were collected 30 minutes prior to the end of the designated time period, washed, dechorionated, hand-sorted and held until the end of the time period. Identical numbers of embryos were homogenized in reducing buffer and heated to 95°C prior to resolution on 10% or 4-12% NuPage gels. Identical 'embryo equivalents' were loaded per lane (GFP=5, Ttv=20, Dlp=40, Dally-myc=40, Sfl=60). Antibody concentrations were: 1:1000 for anti-Myc, anti-Sfl and anti-Dally; 1:10,000 for anti- β Gal; 1:7000 for anti-Ttv; and 1:500 for anti-GFP JL-8.

Heparitinase digests, 3G10 staining

For westerns using 3G10, 100 dechorionated embryos were homogenized in 60 μ l of heparitinase buffer (0.1 M sodium acetate) containing protease inhibitors but lacking calcium. Lysates were treated with 0.25 mU of heparitinase III (Seikagaku Corporation) for 1 hour at 37°C before the digestion was terminated by addition of 25 μ l 4 \times sample buffer, 10 μ l β -mercaptoethanol and incubation at 95°C. Forty embryo equivalents were loaded per lane.

RT-PCR

RNA prepared from bleach-dechorionated 2- to 4-hour embryos and 0- to 3-hour unfertilized eggs (RNA-Easy, Qiagen) was used for RT-PCR (OneStep RT-PCR, Qiagen). PCR products were analyzed on a 0.7% agarose gel. Primer sequences were: Sgl-F1-TGAACACGCCACAAAACCC, Sgl-B2-TCGCCACCTCGGAAACACT, Frc-F2-ACGGTGGTAAACAAGAC-GGTGC, Frc-B9-CAGGCAGAAACATAAACAGCGAG, Oxt-F4-TGGTAATCACACGGCGAACG, Oxt-B1-GGAACTTGGACTCCAA-

CTCCAGC, GalTI-F2-CGAGACCGATTTGAGGAACTCC, GalTI-B1-GTGTCCCCTTACGATGATAGC, GalTII-F11-GCTGAAAAGTGGACCGACGATACC, GalTII-B11-CCTCATCATCTGCCATTGC, GlucaTIA-F1-TTCCTCGTGGTGGCTGATGATGG, GlucaTIA-B2-CGGCTCTATC-CAAAAGGTTTCTGAC, GlucaTIB-F4-GCAAATCCCAGAACTA-ACCCGTC, GlucaTIB-B6-AACCCAGGAGTCCAGGAATGCTAC, GlucaTIC-F2-GCATCCTCCATCTCCTCCATTC, GlucaTIC-B3-TCGTC-TTGTGGCATCCTCG, Botv-F1-GCTGCTGATGCTGCTGTTTCTC, Botv-B1-ACCCTTTTCGCTGAACGCTATG, Ttv-F23-AAGCAGCCTG-GTTTGGAAACG, Ttv-B22-GCATGCCTGAATCTCATCGTAG, Sotv-F1-CCGTAGCAGTCGTCAGTGAATAC, Sotv-B7-TCAGGAA-ACTCTCGCCAAACTTC, Dlp-F1-ACAGCAACAACAAGTCCCG, Dlp-B2-TGCCAGGATTCCAGACATACG, Dally-F4-AGTGGGACT-TACAGCGAAAAAGG, Dally-B4-CGGGGAACAACACTGAACAAAGA-AG, Sfl-F6-AATGGGAATGGGAACGGAAG, Sfl-B10-CCACAAA-AACGAGGACCTTGG, DTtv-5UTR-F1-CAACCGGTGGCAGTGT-TGCTTAAAG, DTtv-5UTR-F2-CAACTGCGCAGCAACTAGTGT, DTtv-5UTR-R1-GGCCTGCATTTTGTGGTTTGTAG, Ttv-F1STOPAGEI-GA-AATACCGGTAGATCGAGTTGGTC, Ttv-R1ENDSTUI-CCTGAAT-TCATCTGCAATCGTGTATATTG, DallyF1-CTGCTGCACAATGCCAC, DallyR1-CTGGACACTGCCAATTC, DallyF2-CAAGTTCTAGGAGCG-AAAC, DallyR2-GTTGCTCATCGAGAAG.

Cell culture, transient transfections and luciferase assays

The pRSTF vector contains an SV40 promoter for expression in cell culture and a T7 promoter that allows in vitro transcription (Jang et al., 2004). For reticulocyte assays, 0.5 μ g of pRSTF dual luciferase vector constructs containing either CV or Ttv UTRs were added to 20 μ l of TNT reticulocyte lysate T7 quickmaster mix (Promega) containing 0.5 μ l of 1 mM methionine. Reactions were carried out at 30°C for 90 minutes, then 5 μ l of the reaction was assayed using the Dual Luciferase substrate (Promega). *Drosophila* S2 cells were maintained in 1 \times Schneider's medium (GIBCO) supplemented with 10% FBS and 1% penicillin/streptomycin. Sixteen to 24 hours prior to transfection, 3 \times 10⁶ cells were seeded per well into 6-well plates. For transient transfections, 100 ng of the dicistronic constructs were incubated with Effectene (QIAGEN). Forty-eight hours post-transfection, cells were lysed in 1 \times Passive Lysis Buffer (Promega) and assayed for luciferase activities. Readings for Firefly luciferase were normalized to Renilla luciferase numbers for all samples, and average values are represented as fold elevation over the luciferase values of pRSTF lacking an IRES.

Transgenic constructs

Germline transformation constructs were generated in Gateway vectors developed by Terence Murphy and obtained from the *Drosophila* Genomics Resource Center. Multiple independent lines analyzed for each construct showed identical temporal regulation. The *ttv* 5' UTR was PCR-amplified from genomic DNA using primers DTtv-5UTR-F1 and DTtv-5UTR-R1. A 5' UTR lacking all upstream AUGs was generated using the DTtv-5UTR-F2 and DTtv-5UTR-R1 primers. PTVW UAS-Venus (enhanced GFP) expression constructs were generated using the Gateway system to insert these 5' UTRs upstream of the Venus open-reading frame to produce long (5'ttv-GFP) and short (Δ UAG-GFP) versions. The primers Ttv-F1STOPAGEI and Ttv-R1ENDSTUI were used to generate the 3' UTR PCR products incorporating *AgeI* and *StuI* sites, respectively. The Δ UAG-GFP plasmid was cut with *AgeI* and *StuI* to remove the Gateway 3' UTR, which was replaced with the PCR-amplified 3' UTR from *ttv* to create Δ UAG-GFP-ttv3'. In addition, the portion of the 5' UTR containing upstream AUGs was excised from the 5'ttv-GFP plasmid using *NdeI* and *SpeI*, and was inserted into Δ UAG-GFP-ttv3', also cut with *NdeI-SpeI*, to create a GFP expression construct that contains full-length *ttv* 5' and 3' UTRs (ttv5'-GFP-ttv3'). Control GFP constructs lacking *ttv* sequences (-GFP-) were generated by isolating an *NdeI-AgeI* fragment containing the Gateway 5' UTR and GFP coding sequence from the DGRC 1091 vector (PTVW), and directionally cloning into Δ UAG-GFP cut with *NdeI-AgeI* to replace the GFP and the *ttv* UTR. To generate GFP constructs containing only the *ttv* 3' UTR (GFP-3'ttv), the *NdeI-AgeI* fragment from 1091 PTVW was directionally cloned into *NdeI-AgeI* digested Δ UAG-GFP-3'ttv (see Fig. S1 in the supplementary material).

RNA folding

Secondary structure predictions and free energy values for *Drosophila* were obtained using MFOLD (Zuker, 2003) with the temperature set at 25°C. Transcript accession numbers are listed in Table S1 in the supplementary material. The number of upstream AUGs and the ΔG values represent minimal estimates as the 5' extent of many of the transcripts has yet to be experimentally determined.

RESULTS

Loss of HSPGs differentially affects embryonic Hh, Wg and BMP signaling

During embryonic development, the absence of maternal and zygotic activity of *ttv*, *sov*, *botv* or *sulfateless* (*sfl*; an N-deacetylase/N-sulfotransferase, NDST) which encode GAG chain synthetic enzymes, results in segmental loss of naked cuticle along the anteroposterior (AP) axis, which is indicative of impaired *hh* or *wg* signaling (Fig. 1A-D) (Bornemann et al., 2004; Han et al., 2004; Lin and Perrimon, 1999; Takei et al., 2004; The et al., 1999). Similar defects are seen in embryos lacking *sugarless* (*sgl*) and *fringe connection*, which encode, respectively, a UDP-glucose dehydrogenase and a nucleotide sugar transporter necessary for GAG chain precursor synthesis (Binari et al., 1997; Goto et al., 2001; Hacker et al., 1997; Haerry et al., 1997; Selva et al., 2001). Remarkably however, *ttv*, *sfl*, *sgl* and other GAG synthesis mutants show no defects in DV patterning, such as loss of amnioserosa, expansion of ventral denticle belts or reduction in filzkörper (Fig. 1C,D; data not shown) (Haerry et al., 1997). This result is unexpected as HSPGs are required for Dpp signaling in imaginal discs, and disruption of the BMP activity gradient essential for specification of dorsal cell fates in early embryogenesis results in ventralization.

One potential explanation for the insensitivity of embryonic DV patterning to germline loss of HSPGs could be rescue by a somatic source. The BMP activity gradient is generated in the perivitelline fluid surrounding the embryo, which is supplied by somatic follicle cells during oogenesis (reviewed by O'Connor et al., 2006). As glypicans can transfer between different cells, and proteoglycan ectodomains can be shed from the cell surface (Kreuger et al., 2004), HSPGs contributed by follicle cells could potentially rescue embryonic DV patterning even if the germline lacks GAG synthesis. To examine this possibility, we generated flies lacking Ttv activity in both the ovarian germline and the soma using a temperature-sensitive GAL4-GAL80 system to conditionally rescue larval lethality and recover homozygous *ttv* adults. Rescued females were transferred to 25°C, the GAL80 permissive temperature, to block Ttv production in all tissues. Homozygous mutant embryos derived from such Ttv-depleted mothers displayed segmentation defects similar to germline clones, but showed no signs of ventralization (Fig. 1E). Eggs laid by Ttv-depleted mothers were shorter than wild-type or *ttv* germline null eggs, and had reduced opercula reminiscent of eggs laid by females deficient in Dpp signaling in follicle cells (Chen and Schupbach, 2006; Shravage et al., 2007; Twombly et al., 1996), providing evidence that follicle cell GAG synthesis was successfully blocked under these experimental conditions and that BMP activity in follicle cells requires HSPGs (Fig. 1F-H). Taken together, these results demonstrate that BMP signaling is unaffected by an absence of HSPGs in the early embryo, but is sensitive to their loss at other developmental stages.

HSPG synthesis is blocked during early embryonic development

Given that Hh and Wg signaling are impaired in embryos that lack GAG synthesis, we considered the possibility that the differential sensitivity of Dpp could have a temporal basis. The phosphorylated

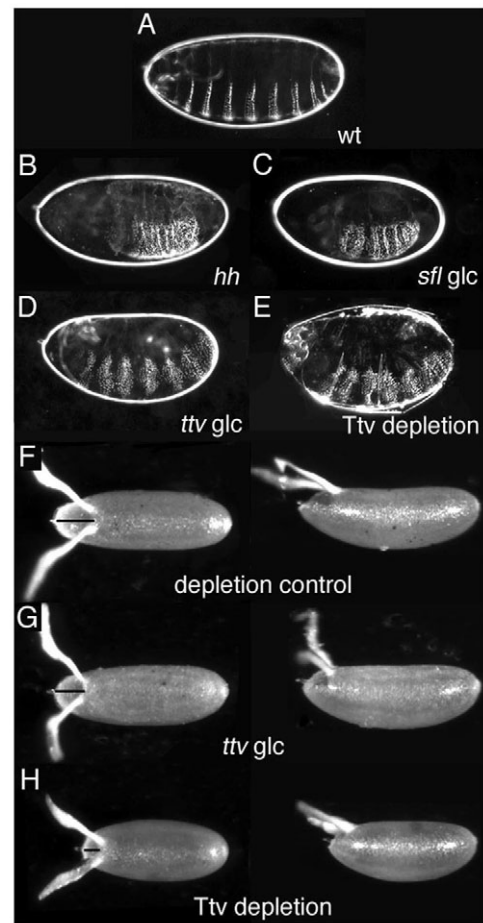


Fig. 1. Embryonic dorsoventral patterning is unaffected by the absence of HSPG synthesis. (A-E) Dark-field images of cuticles from (A) wild-type and (B-E) mutant larvae. (F-H) Dorsal and lateral views of eggs laid by wild-type and *ttv* mothers. Anterior is to the left. (A) The ventral cuticle of wild-type larvae displays a regular segmental pattern of denticle belts alternating with bands of naked cuticle. (B) Larvae mutant for *hh* (as well as *wg*) develop a continuous lawn of denticles due to loss of naked cuticle. (C) *sfl* germline clone (*glc*) larvae lacking maternal and zygotic N-deacetyl/N-sulfotransferase activity show loss of naked cuticle and fused denticles resembling the *hh* mutant phenotype, but no increase in denticle belt width or DV patterning defects. (D) *ttv glc* larvae display a similar phenotype, with no abnormalities in DV patterning. (E) The phenotype of a *ttv* mutant larva from a depleted mother that lacks somatic and germline *ttv* activity resembles *ttv glc* mutants. (F) Paired paddle-shaped dorsal appendages are located posterior to the operculum (line) at the anterior of the wild-type eggshell. (G) Eggshell morphology is unaffected by loss of *ttv* activity in the germline. (H) Eggs laid by Ttv-depleted females are shorter and have a smaller operculum (black bar), phenotypes associated with reduced Dpp signaling activity in follicle cells.

form of Mothers against Dpp (pMad), a direct substrate of the activated Dpp receptor Thickveins, can first be visualized ~2.5 hours after fertilization (mid-stage 5) on the dorsal side of the embryo in a shallow gradient, which rapidly sharpens over the next 30–45 minutes to form a steep gradient with peak levels in the dorsal-most 5–9 cells (Ross et al., 2001; Rushlow et al., 2001). By contrast, Hh and Wg signaling trigger changes in the intracellular localization of their downstream targets Armadillo and Cubitus interruptus, respectively, at a later point, 3 to 4 hours post-fertilization (stages 6–

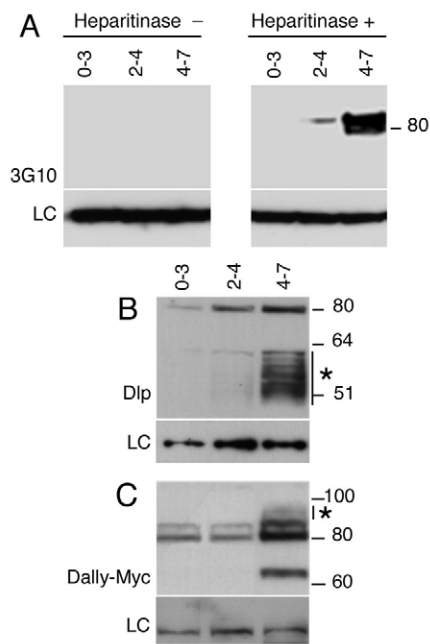


Fig. 2. HSPG GAG chain synthesis is temporally regulated.

(A) Western blots of staged embryonic extracts probed with 3G10 antisera against GAG chain stub epitopes generated by heparitinase III digestion. GAG-modified core proteins are undetectable in 0-3 hour embryo extracts but are seen in 2-4 and 4-7 hour samples. No signal was detected in the absence of heparitinase. LC, loading control (B,C) Staged embryonic extracts were probed with (B) anti-Dlp to detect Dlp and with (C) anti-Myc to detect Dally. *matTub>Gal4* was used to drive UAS-Dlp or UAS-Dally expression maternally and embryonic extracts from the indicated stages resolved on reducing gels. Dlp was detected as a sharp band at 80 kDa, corresponding to full-length protein, and a heterogeneous band between 50 and 60 kDa, which represents a GAG-modified cleavage product. Low levels of full-length Dlp are seen at 0-3 and 2-4 hour extracts. Significant levels of GAG modification are first apparent in 4-7 hour extracts as decreased mobility of the full-length protein (asterisk). The ~65 kDa band is likely to result from processing by protein convertases, similar to vertebrate glypicans.

10) (Motzny and Holmgren, 1995; Peifer et al., 1994). To examine whether GAG modification could be developmentally regulated, we probed western blots of staged embryonic extracts with 3G10 antisera that recognize stub epitopes generated by heparitinase digestion, thus identifying all GAG-modified HSPGs (David et al., 1992). We found that no signal could be detected in 0-3 hour embryos, although several bands were present in extracts from later stages, indicating that GAG modifications are absent during early development (Fig. 2A). Furthermore, no signal was detected in the absence of heparitinase treatment, confirming the specificity of the antisera. Because 3G10 does not reveal the glycosylation status of individual core proteins, we next examined GAG addition to the glypican core proteins, Division abnormally delayed (Dally) and Dally-like (Dlp), that participate in Hh, Wg and Dpp signaling (Han et al., 2005; Jackson et al., 1997; Kirkpatrick et al., 2004; Kreuger et al., 2004). UAS-Dlp and epitope-tagged UAS-Dally were maternally loaded into oocytes using the strong maternal *matTub>Gal4* driver. Embryonic extracts prepared from the indicated stages were resolved on reducing gels and probed to detect

Dlp and Dally core proteins (Fig. 2B,C). A sharp band at 80 kDa corresponding to maternally driven full-length Dlp lacking GAGs was detected at all time points. In addition, high levels of a GAG-modified cleavage product that retains the antigenic epitope and migrates as a heterogeneous band between 50 and 60 kDa could be seen in 4-7 hour extracts and, at lower levels, in 2-4 hour samples (Fig. 2B). The 50-60 kDa band collapses to a band of 49 kDa upon heparitinase treatment and is recognized by 3G10, confirming its identity as a GAG modified product (data not shown). Thus, although full-length Dlp was detected at low levels in 0-3 hour extracts, GAG modifications were essentially absent at this time. GAG modifications were first observed in 2-4 hour extracts and were dramatically upregulated in 4-7 hour embryos (Fig. 2B). Modification of the related glypican Dally followed a similar timeline. Full-length epitope-tagged Dally was visible as an ~80 kDa doublet in 0-3 and 2-4 hour extracts (Fig. 2C). Significant levels of GAG modification were first apparent in 4-7 hour extracts as a broad band migrating more slowly than the full-length protein (asterisk in Fig. 2C). Interestingly, an ~65 kDa cleavage product was specifically detected in 4-7 hour extracts, indicating that Dally may undergo processing by protein convertases, similar to vertebrate Glypican 1, 3 and 4 (Song and Filmus, 2002). In conclusion, GAG modifications of both glypicans are either absent or present at very low levels during the period when the Dpp activity gradient is established, and only become abundant concurrent with the earliest requirement for Hh and Wg.

GAG enzyme expression is regulated at the translational level

A potential explanation for the delayed onset of glycosylation could be that mRNA for one or more key enzymes is only expressed zygotically, following the initiation of embryonic transcription between 1.5 and 2.5 hours after fertilization. We therefore used RT-PCR to determine whether transcripts for any GAG pathway components were absent in unfertilized eggs, which contain only maternally provided mRNAs. Positive signals were obtained for all enzymes, as well as for the core protein Dlp, indicating that the temporal control of GAG synthesis does not rely on developmentally regulated transcription (Fig. 3A).

To determine whether GAG synthesis was regulated through a post-transcriptional mechanism, we used antisera that recognize endogenous Sfl (Yano et al., 2005) and Ttv (The et al., 1999) to probe developmental western blots. Despite the fact that both genes are maternally transcribed (see Fig. 3A), significant levels of protein expression were only detected after 3 hours of development (Fig. 3Bi,ii; compare 0-3 and 2-4 hour lanes). The minimal level of Sfl and Ttv in 0-3 hour samples indicates that the proteins are not deposited into the egg, and that the maternally provided transcripts encoding Sfl and Ttv are not translated in early embryos. Recent studies have suggested that, in *Drosophila*, 20-27% of maternal mRNAs are degraded following egg activation (Arbeitman et al., 2002; Tadros et al., 2007). This raised the possibility that maternal *sfl* and *ttv* mRNA might not contribute to the dramatic increase in the level of the corresponding proteins in the time period between 3 and 7 hours post-fertilization. We therefore probed western blots of extracts from unfertilized eggs to determine whether maternal transcripts contribute significantly to GAG enzyme levels. We found that Sfl and Ttv are essentially absent in 0-3 hour eggs, but that their levels increase dramatically at later stages, similar to what was observed in embryos (Fig. 3Biii,iv). As zygotic transcripts are absent in unfertilized eggs, maternal mRNA is responsible for all of the output. This result suggests that the protein expressed from maternal

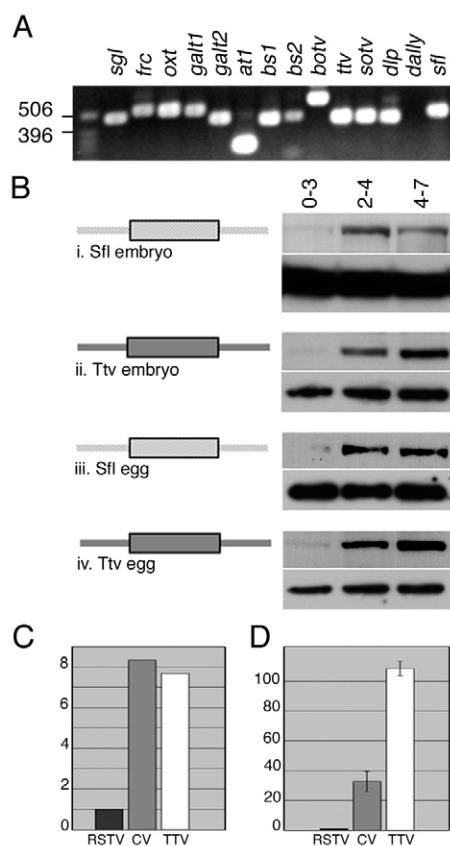


Fig. 3. GAG enzyme synthesis is post-transcriptionally regulated. (A) Transcripts for enzymes involved in HSPG GAG chain synthesis are maternally provided. Template RNA isolated from unfertilized eggs supports the generation of RT-PCR products for all enzymes involved in HSPG GAG chain synthesis. The UDP glucose dehydrogenase Sgl and the Glucuronic acid transporter Fringe connection (Frc) act in the synthesis of GAG chain building blocks. Peptide-*o*-xylosyltransferase (Oxt), Galactosyltransferase I and II (GalT1, GalT2), and Glucuronyltransferases AT-1, BS1 and BS2 synthesize the tetrasaccharide linker; the polymerases Botv, Ttv and Sotv are required for chain elongation. The glypicans Dlp and Dally encode the core protein substrate for GAG chain addition, and the N-deacetylase-N-sulfotransferase Sfl modifies sugar residues on the polymerised GAG chains. Dally mRNA is undetectable in eggs although an RT-PCR product can be generated using RNA from 0-24 hour embryos. To rule out amplification of a DNA template, primer pairs for all genes except GalT2 (which lacks an intron) span one or more introns. (B) Western blots of staged embryonic extracts were probed to visualize expression of the indicated proteins. Loading controls are shown below each panel. (i) Endogenous Sfl is absent from 0-3 hour embryos and is first detected in 2-4 hour extracts. (ii) Antisera against Ttv reveal that expression of the endogenous protein follows a similar temporal profile. (iii) In unfertilized eggs, Sfl is barely detectable at 0-3 hours but is robustly expressed in 2-4 and 4-7 hour samples. (iv) Ttv expression in unfertilized eggs mirrors its expression in embryos. (C,D) The *ttv* 5' UTR shows IRES activity in (C) reticulocyte lysates and (D) *Drosophila* S2 cells. Luciferase assays were carried out on reticulocyte in vitro translation extracts or lysates from cells transfected with an unmodified bicistronic vector (RSTV), or a vector with the CV 5.1 IRES (CV) or the *ttv* 5' UTR (TTV). The ratio of Renilla to Firefly luciferase was calculated for each sample and the data represented as fold amplification relative to the values for the empty pRSTF vector, set at 1. In in vitro translation extracts, the *ttv* 5' UTR confers a 7.5-fold increase in firefly luciferase expression, comparable to the 8-fold increase generated by the CV IRES (average of two assays). In cultured cells, the *ttv* UTR is more than three times more effective than the viral IRES, and results in ~108-fold stimulation compared with empty vector. The graph represents the average values and standard error from five independent transfection assays.

transcripts represents a significant contribution to embryonic GAG synthetic activity. Moreover, it is consistent with the fact that maternal *ttv* or *sfl* activity is sufficient to allow the survival of homozygous animals to larval third instar. Taken together, these data indicate that maternal mRNA enhances the level of Sfl and Ttv expression, and that translational control of essential synthetic enzymes may explain the absence of GAG modification in the early embryo.

Analysis of the *ttv* and *sfl* 5' UTRs showed that, at 621 and 1261 nucleotides, they are significantly longer than the average *Drosophila* 5' UTR (256 bases) (Misra et al., 2002) and contain numerous upstream AUG codons (15 and 19, respectively) that would be expected to interfere with translation by blocking ribosomal scanning. In addition, these 5' sequences are predicted to form complex secondary structures with ΔG s of -129 and -265 kcal, well above the threshold (-50 kcal) for interference with translation (Zuker, 2003) (see Table S1 in the supplementary material). These features are consistent with the presence of IRESs that facilitate cap-independent assembly of the translation complex at downstream initiation codons (Hellen and Sarnow, 2001). To determine whether *ttv* mRNA contains an IRES, we cloned the 5' UTR into the intercistronic region of the bicistronic reporter pRSTF (Jang et al., 2004). In this vector, translation of the upstream Renilla luciferase open reading frame (ORF) is directed by cap-dependent ribosome scanning, whereas a stable stem loop blocks expression of the downstream firefly luciferase unless an IRES is present. Insertion of *ttv* 5' UTR sequences consistently resulted in high levels of firefly luciferase translation compared with pRSTF alone (Fig. 3C). In reticulocyte lysates, inclusion of the *ttv* 5' UTR in T7 promoter-driven transcripts resulted in 7-fold higher levels of firefly luciferase when compared with the unmodified vector, equivalent to the

stimulation achieved with the well-characterized coxsackievirus (CV) IRES 5A (Jang et al., 2004) (Fig. 3C). To test if the *ttv* IRES functions in a physiological system, we carried out similar experiments by transfection in *Drosophila* S2 cells. The presence of the *ttv* 5' UTR resulted in efficient stimulation of firefly luciferase, with expression levels more than twice that of the viral construct, thus demonstrating its IRES activity (Fig. 3D).

Developmental regulation of *ttv* in vivo requires both 5' and 3' untranslated regions

To determine whether other *ttv* non-coding sequences play a role in regulating IRES-dependent translation, we first used western blots to examine the consequences of deleting both the 5' UTR region containing upstream initiator codons and the 3' UTR, on the Ttv expression profile. We found that maternally expressed UAS constructs lacking these regions directed the translation of Myc-tagged Ttv prematurely in 0-3 hour embryos (Fig. 4Ai, upper band). By contrast, the endogenous protein was not expressed until 3 to 4 hours after fertilization (Fig. 4Ai, lower band), demonstrating that either the leader or trailer sequences, or both, were necessary for *ttv* temporal regulation. Consistent with this idea, a UAS-GFP transgene lacking *ttv* flanking sequences also directed protein expression at all stages (Fig. 4Aii). Next, to determine whether these non-coding sequences were sufficient for regulated expression, we generated transgenic lines in which GFP coding sequences were flanked by 5', 3' or both *ttv* UTRs. We found that maternally driven UAS-GFP expression was blocked in early embryonic stages only when both UTRs were present (Fig. 4Aiii). By contrast, transcripts lacking either the 3' (5'*ttv*-UAS-GFP) or 5' (UAS-GFP-3'*ttv*) UTRs were incorrectly regulated and resulted in premature GFP expression (Fig. 4Aiv,v).

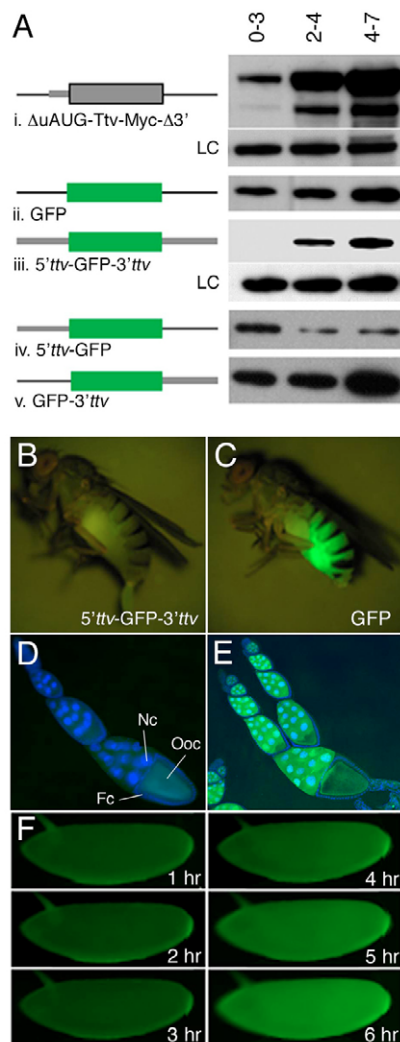


Fig. 4. Delineation of mRNA cis-elements that regulate *ttv* expression. (A) Western blots of staged embryonic extracts from flies transgenic for the indicated constructs were probed to visualize the expression of Ttv or GFP. LC, loading control. (i) Truncation of the 5' UTR to eliminate upstream AUG sequences and removal of the 3' UTR permits the translation of maternally loaded Ttv-myc transcript in 0-3 hour embryos (upper band); the endogenous protein (lower band) is not expressed at this time. (ii-v) *matTub>Gal4* was used to drive the expression of UAS constructs in which the GFP coding sequence was flanked by the indicated regions of *ttv*. (ii) GFP expression was detected in 0-3 hour extracts in the absence of *ttv* regulatory sequences, as well as when only 5' (iv) or 3' (v) UTRs were present. (iii) By contrast, GFP expression was blocked in early embryos and initiated at ~3 hours, similar to endogenous Ttv, when the ORF was flanked by both 5' and 3' UTR sequences. (B-F) Translational regulation visualized in live animals. (B) GFP fluorescence is absent from a female transgenic for a *matTub>Gal4* driver and a construct in which GFP is flanked by *ttv* 5' and 3' UTRs. (C) By contrast, ovarian expression results in bright fluorescence in a female transgenic for *matTub>Gal4* and a GFP construct that lacks *ttv* UTRs. (D,E) Merged images showing DAPI-stained nuclei and GFP fluorescence in egg chambers. (D) Egg chambers from an animal carrying *matTub>Gal4* and the reporter construct with *ttv* 5' and 3' UTRs, showing the absence of GFP expression and only yolk autofluorescence. (E) By contrast, a reporter that lacks *ttv* UTRs generates high levels of GFP expression in nurse cells (Nc) and oocytes (Ooc). Note that expression cannot be detected in follicle cells (Fc). (F) Time-lapse series of a 5'ttv-UAS-GFP-3'ttv embryo shows that significant GFP fluorescence develops only after three hours post-egg laying.

Developmentally regulated translation was also apparent in live animals. GFP fluorescence was absent from females transgenic for the maternally driven 5'ttv-UAS-GFP-3'ttv construct, and no GFP expression could be detected in somatic or germline cells in the ovary (Fig. 4B,D). In embryos derived from these females, GFP expression was detected only after 4 hours of development (Fig. 4F). By contrast, females expressing transgenes lacking either 5', 3' or both *ttv* UTRs showed high levels of fluorescence due to the accumulation of GFP in nurse cells and the oocyte (Fig. 4C,E; data not shown). Importantly, all transgenes tested were inactive in the absence of a GAL4 driver, ruling out the possibility that a cryptic promoter in the 5' UTR (rather than an IRES) is responsible for the GFP expression (data not shown). Collectively, these data establish that the *ttv* 5' UTR contains a temporally regulated IRES, and that *ttv* 5' and 3' UTRs act in concert to confer developmental regulation on adjacent coding sequences by preventing their translation during early embryogenesis, while permitting expression at later stages.

Exogenous heparin disrupts BMP signaling and dorsal embryonic patterning

The block to GAG synthesis coincides with the period when the BMP gradient is established in the embryo, raising the issue of whether the premature expression of HSPGs disrupts DV patterning. As misexpression of HSPGs in the embryo would require releasing multiple genes from translational control, it is not technically feasible to test this possibility directly. We therefore addressed this issue by injecting heparin (which mimics some HSPG functions) into the embryonic perivitelline space and assaying its effect on BMP activity. A *Kruppel (Kr)-lacZ* reporter transcribed in the dorsal-most amnioserosa cells was used as a readout for peak levels of BMP signaling. We found that heparin-injected embryos showed a dramatic reduction or loss of *Kr-lacZ* expression, as well as morphological defects typical of ventralization, such as a prominent cephalic furrow and defective germ band extension. Together, these phenotypes indicate that cell fate specification along the DV axis is severely disrupted by heparin. By contrast, control embryos injected with buffer alone showed normal *lacZ* expression and morphology (Fig. 5A,B). Although this result is consistent with direct inhibition of Dpp signaling by heparin, it left open an alternative explanation. Heparin is known to bind and activate the serine-protease inhibitor (serpin) antithrombin that inactivates proteases in the blood-clotting cascade (Huntington, 2003). Ectopic heparin could conceivably sequester or inhibit an endogenous serpin, and thus promote the serine protease cascade in the perivitelline space that culminates in nuclear localization of the morphogen Dorsal (Dl) on the ventral side of the embryo (Moussian and Roth, 2005). Because Dl represses *dpp* transcription in ventral cells (thus restricting its expression to the dorsal half of the embryo), ectopic activation of Dl would lead to the loss of *dpp* expression and elimination of the BMP activity gradient. To establish that Dpp signaling is directly inhibited by heparin rather than affected indirectly through the Dl pathway, we injected heparin into *dl⁻* embryos in which *dpp* is expressed ubiquitously and *Kr-lacZ* is activated throughout the DV axis (Fig. 5C). We found that heparin strongly suppressed reporter expression in *dl⁻* embryos as well, demonstrating that heparin acts downstream of *dl* to interfere with Dpp signaling or stability (Fig. 5D). By contrast, both segmental expression of a *wg-lacZ* reporter and embryonic morphology along the AP axis were unaffected by heparin injection, indicating that AP patterning that depends on Wg/Hh activity is not compromised at the heparin levels that disrupt BMP signalling (Fig. 5E,F). Collectively, these results suggest that heparin specifically affects

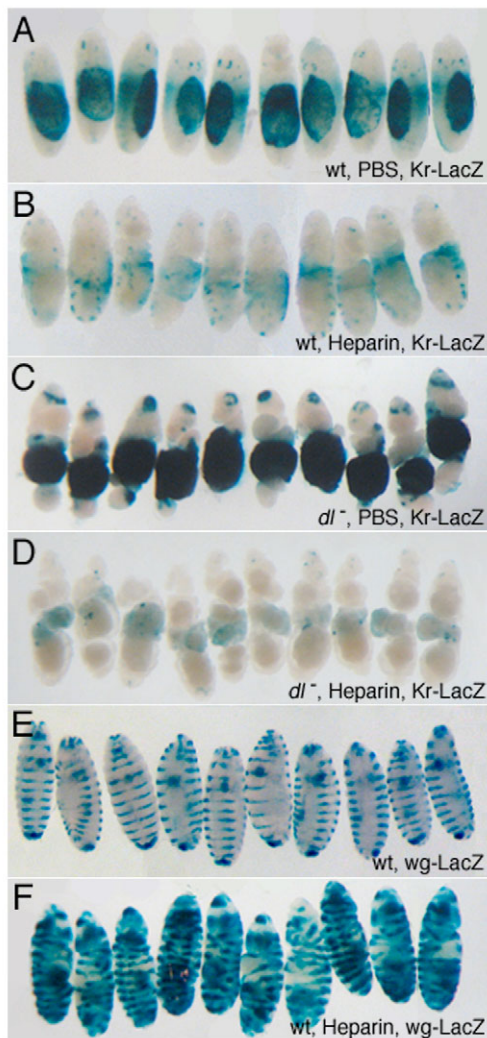


Fig. 5. Perivitelline injection of heparin inhibits Dpp signaling and disrupts embryonic dorsoventral patterning.

Heparin/PBS solution was injected into the perivitelline space of 1-2 hour embryos to achieve the specified final concentrations (see Materials and methods). Embryos were allowed to develop for 21 hours before fixation and staining. The dorsal-most embryonic tissue, the amnioserosa, is marked by the expression of a *Kr-lacZ* transgene (A-D), which provides a readout of alterations in DV patterning. Representative embryos are shown; the total sample size is indicated in parenthesis for each genotype. (A,B) Injection of heparin at 1.5 $\mu\text{g/ml}$ (B) leads to a reduction in *Kr-lacZ* expression (A, $n=136$; B, $n=114$) and morphological defects typical of loss of dorsal cell fates, such as expansion of the cephalic furrow. (C,D) In *dl* embryos, ubiquitous *dpp* expression results in ventral expansion of reporter expression (C, $n=40$). Injected heparin (10.5 $\mu\text{g/ml}$) inhibits reporter expression in embryos lacking *dl* activity, demonstrating that heparin directly interferes with BMP signaling (D, $n=37$). (E,F) By contrast, embryonic morphology along the AP axis and segmental expression of a *wg-lacZ* reporter (E) are unaffected by heparin (1.5 $\mu\text{g/ml}$; F, $n=121$). This indicates that AP patterning and Wg/Hh activity are not compromised at heparin levels that disrupt BMP signaling, thus providing a control for specificity. Expansion of *wg-lacZ* stripes laterally (F) so that they encircle the embryo reflects cell fate changes resulting from ventralization.

BMP signaling in the early embryo, and that delayed GAG synthesis may be necessary to enable BMP gradient formation that would otherwise be disrupted by the presence of HSPG GAG chains.

DISCUSSION

There is extensive evidence that the structurally complex, negatively charged GAG side chains of HSPGs are crucial for signaling by diverse secreted ligands, including BMPs. Although the distribution of HSPGs is generally assumed to be ubiquitous, our data showing that GAGs are absent in the first three hours after egg laying but are rapidly synthesized thereafter demonstrates that HSPG expression can, in fact, be precisely temporally regulated. We have shown that this regulation results from an absence of enzymes essential for HSPG synthesis, owing to a block to their translation. Furthermore, our data strongly support the notion that the translational control mechanism involves the use of developmentally regulated internal ribosome entry sites (IRESs) in mRNA 5' UTRs. Translationally blocking GAG synthesis may enable generation of the Dpp/Scw activity gradient in the absence of GAGs, while permitting rapid GAG synthesis from the abundant maternal mRNA supply coincident with the expression of Hh and Wg, which require HSPGs for signaling, just an hour later. Consistent with this idea, we have shown that injection of heparin into early embryos compromises BMP signaling. This finding was unexpected, as HSPGs have been shown to promote BMP signaling in the wing disc (Bornemann et al., 2004; Fujise et al., 2003; Han et al., 2004; Takei et al., 2004). However, there are other instances in which HSPGs have been shown to have context-specific effects. For example, Dally enhances Dpp activity in the eye, antennae and genitalia, but reduces Dpp signaling in the developing wing (Jackson et al., 1997). Furthermore, Dlp has been shown to negatively affect the expression of high-threshold Wg targets at short range, and to enhance the expression of low-threshold targets at long range (Baeg et al., 2004; Kirkpatrick et al., 2004). The differential effects of HSPGs highlighted in this study may reflect the fact that the BMP gradients in the embryo and the wing are established through significantly different mechanisms. In embryos, *dpp* is transcribed uniformly in dorsal cells and a short-lived gradient of BMP activity is generated through rapid redistribution of the ligand within this space. By contrast, in the wing disc, *dpp* expression is spatially restricted, and a stable concentration gradient forms over several hours. A number of proteins necessary for formation of the embryonic gradient have been shown to bind HSPGs. These include Dpp itself, a second BMP Screw, and the extracellular proteins Short Gastrulation (Sog) and Twisted Gastrulation, which modulate ligand activity in a context-dependent fashion (Groppe et al., 1998; Mason et al., 1997). In vertebrates, HSPG binding to the Sog homolog Chordin, limits its diffusion and enhances its ability to antagonize BMP signaling (Jasuja et al., 2004). Thus, premature expression of HSPGs in the *Drosophila* embryo could reduce mobility of the ligands and/or Sog/Tsg complexes, thereby affecting facilitated transport and compromising DV patterning. In addition, interaction of HSPGs with BMP2/BMP4 has been implicated in increased ligand degradation and reduced signaling range (Degnin et al., 2004; Ohkawara et al., 2002). Therefore, HSPGs could disrupt DV patterning by affecting several parameters crucial for establishment of the embryonic BMP morphogen gradient.

Several lines of evidence suggest that the regulated expression of *sfl* and *ttv* results from the use of IRES elements and sequences in the 3' UTR. First, the 5' UTRs of both genes contain multiple AUG codons that are flanked by purines at position -3, and are thus in the optimal context for translation initiation. Second, we have demonstrated that the *ttv* 5' UTR can mediate translation of a downstream ORF in both in vitro translation assays and cell culture. Furthermore, transgenes lacking upstream AUGs and 3' UTR sequences do not display regulated expression, although the

5' and 3' UTRs are sufficient to confer regulation on the heterologous GFP ORF. This mechanism is distinct from the regulation of 'masked' maternal mRNAs, for genes such as *Drosophila bicoid* (*bcd*) and *Toll* (*Tl*), which are quiescent until egg activation triggers polyA tail extension and enables their translation (Stebbins-Boaz and Richter, 1997; Tadros and Lipshitz, 2005). Transcripts for *sfl* lack consensus sites for cytoplasmic polyadenylation, and we do not detect an increase in *ttv* polyA tail length in activated eggs using polyA test (PAT) assays (D.J.B., unpublished). Furthermore, *Ttv* and *Sfl* are expressed significantly later than *Bcd* and *Tl*, which are initially detected ~1-2 hours after fertilization, indicating that their translation is not co-ordinately regulated (Driever et al., 1990; Schisa and Strickland, 1998). Our data are also inconsistent with miRNA-based regulation, which occurs primarily through 3' UTRs, as both 5' and 3' *ttv* sequences are necessary to inhibit expression. The fact that the expression of *Sfl* and *Ttv* is translationally controlled both in unfertilized eggs and embryos (see Fig. 3B), and that release of the translational block occurs over the same temporal period, is significant. This finding indicates that translational inhibition, as well as its relief, can be effected solely by maternally provided factors. Because egg activation in *Drosophila* occurs independently of fertilization, it could provide the trigger that lifts the block to HSPG mRNA translation with similar timing in eggs and embryos (Heifetz et al., 2001). Analysis of other GAG pathway enzymes reveals multiple upstream start codons in the 5' UTRs of 3-*O*-sulfotransferase, 6-*O*-sulfotransferase, C5 epimerase and *Dlp* (18, 6, 5 and 3, respectively), suggesting that additional components in the pathway may be similarly regulated to ensure their concurrent expression and enhance the tight temporal control of HSPG synthesis. Although *dally* mRNA is absent from unfertilized eggs (Fig. 3A, see also Fig. S1 in the supplementary material), the transcript contains four upstream AUGs and its expression may be post-transcriptionally regulated (Tsuda et al., 2001), raising the possibility that this mechanism could play a role in modulating HSPG function at other developmental stages.

Germline clonal analysis has shown that embryos laid by mothers mutant for several GAG synthetic genes, including *ttv* and *sfl*, can be rescued paternally by wild-type sperm (Perrimon et al., 1996). These data establish that both zygotic and maternal expression of the genes contributes to their activity. In addition, the data raise the question, why is it necessary to maternally load translationally blocked transcripts, rather than to transcribe them zygotically prior to the onset of Wg and Hh signaling? One potential explanation could be to ensure a rapid initiation of GAG synthesis. The time lag inherent in expression of large loci, such as *ttv* and *sfl*, that span 50-60 kb is likely to be significant given constraints imposed by the fast pace of *Drosophila* embryogenesis. This could be particularly critical if multiple components in the pathway are regulated in a similar fashion. Importantly, the UTRs of GAG-synthetic enzymes in other organisms also exhibit hallmarks suggestive of translational control. The 5' UTRs of the mouse orthologs of *Sfl* (NDST1-NDST4) contain multiple initiator codons and have been shown to have IRES activity (Grobe and Esko, 2002). We find that *Ttv*/*EXT1* and *Sfl* orthologs from *Hydra*, Zebrafish and *Xenopus* also contain highly structured 5' UTRs with several upstream AUGs (see Table S1 in the supplementary material). Because these phylogenetically diverse organisms employ dramatically different developmental strategies, regulation of GAG synthetic activity through translational control is likely to play an important and hitherto unsuspected role in temporal or tissue-specific modulation of growth factor signaling.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/6/1039/DC1>

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