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Protein O-GlcNAcylation Is Required for Fibroblast Growth Factor Signaling in *Drosophila*

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Abstract

Glycosylation is essential for growth factor signaling through N-glycosylation of ligands and receptors and the biosynthesis of proteoglycans as co-receptors. Here, we show that protein O-GlcNAcylation is crucial for fibroblast growth factor (FGF) signaling in *Drosophila*. We found that *nesthocker* (*nst*) encodes a phosphoacetylglucosamine mutase and that *nst* mutant embryos exhibited low amounts of intracellular uridine 5'-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), which disrupted protein O-GlcNAcylation. Nst was required for mitogen-activated protein kinase (MAPK) signaling downstream of FGF but not MAPK signaling activated by epidermal growth factor. *nst* was dispensable for the function of the FGF ligands and the FGF receptor's extracellular domain but was essential in the signal-receiving cells downstream of the FGF receptor. We identified the adaptor protein Downstream of FGF receptor (Dof), which interacts with the FGF receptor, as the relevant target for O-GlcNAcylation in the FGF pathway, suggesting that protein O-GlcNAcylation of the activated receptor complex is essential for FGF signal transduction.

INTRODUCTION

Signaling pathways are part of larger networks within which they interact with each other and with metabolic pathways. The biological functions of these interactions are often complex and remain to be defined. Uridine 5'-diphosphate-*N*-acetylhexosamine (UDP-HexNAc) nucleotides are fundamental metabolites for the glycosylation of proteins and

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SUPPLEMENTARY MATERIALS www.sciencesignaling.org/cgi/content/full/4/204/ra89/DC1

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lipids. UDP-*N*-acetylglucosamine (UDP-GlcNAc) is produced primarily by de novo synthesis from glucose, glutamine, and acetyl coenzyme A (CoA) through the hexosamine biosynthetic pathway (HBP). It is used directly (and through epimerization to UDP-*N*-acetylgalactosamine) by glycosyltransferases associated with the endoplasmic reticulum or Golgi for the synthesis and elaboration of protein N- and O-linked glycans and glycolipids and the synthesis of glycosylphosphatidylinositol (GPI) anchors; it is also used in the cytosol and the nucleus for modification of proteins by O-linked β -GlcNAc (*O*-GlcNAc) (1).

Whereas most protein glycosylation occurs in the lumen of the secretory pathway and leads to the production of relatively large glycans, protein *O*-GlcNAcylation involves the transfer of only a single β -GlcNAc residue to Ser or Thr residues of cytosolic and nuclear acceptor proteins. In this respect, *O*-GlcNAcylation is similar to protein phosphorylation, and, indeed, many substrates are both *O*-GlcNAcylated and phosphorylated (2). Moreover, *O*-GlcNAcylation can regulate the accessibility of phosphorylation sites of target proteins (2, 3). Protein *O*-GlcNAcylation is reversible and the cycle is catalyzed by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA; *O*-glycoprotein 2-acetamido-2-deoxy- β -D-glucopyranosidase) (4, 5). Loss- and gain-of-function studies in fruit flies, zebrafish, and mice revealed that OGT is essential for development (6–10).

Not only OGT but also the HBP itself plays an important role in early development (11). The rate-limiting enzyme in the HBP is GFAT (glutamine:fructose-6-phosphate aminotransferase), which produces glucosamine-6-phosphate, a substrate for glucosamine-6-phosphate acetyltransferase (GNA1, also known as EMeg32) to produce GlcNAc-6-phosphate, which in turn serves as substrate for phosphoacetylglucosamine mutase (PGM3) (fig. S1). Mutations in *PGM3* or *GNA1* cause defects during gastrulation in early mouse development (12, 13), but these defects have not been pinpointed to a discrete molecular pathway. In contrast, embryos that lack complex *N*-glycans undergo gastrulation and die only later in development (14), suggesting that the defects in mutations of HBP enzymes affect gastrulation by interfering with a distinct pathway.

Here, we showed that in *Drosophila*, loss-of-function mutations in the *nst* gene, which encodes PGM3, blocked fibroblast growth factor (FGF) signaling during embryonic mesoderm and tracheal development. In *nst* mutants with an 80% reduction in the amount of UDP-GlcNAc, *O*-GlcNAcylation was compromised. We demonstrated by genetic, biochemical, and small-compound inhibitor studies that protein *O*-GlcNAcylation was essential for FGF-dependent morphogenesis and differentiation. These results provide evidence for a critical signaling node between the HBP and the FGF pathways.

RESULTS

The *nst* mutation affects FGF signaling

Previous pan-genomic screens indicated that, in *Drosophila*, most gene products required for FGF signaling in early embryos are provided maternally (15). We, therefore, performed a female germline mosaic screen to identify maternal genes involved in FGF-dependent mesoderm migration (15, 16). The screen identified a range of mutations affecting genes involved in glycosylation, including the previously characterized mutations *sugarless*, *fringe connection*, *tout velu*, and *sulfateless*. This class of mutations exhibits a known stereotypic pattern of defects in cell signaling affecting Wingless (Wg), Hedgehog (Hh), and FGF signaling (15). We identified one mutation, *nst*, which showed phenotypes characteristic of mutations in the FGF receptors (FGFRs) (Fig. 1, A to E). Embryos maternally and zygotically mutant for *nst* (*nst*^{MZ}) exhibited defects in mesoderm layer formation during gastrulation that were similar to those observed in embryos with mutations in the gene encoding the FGFR Heartless (Htl) (Fig. 1A). Tracheal development, which depends on the

FGFR Breathless (Btl), was also affected (Fig. 1B); similar to *btl* mutants, the generation of the dorsal tracheal trunk was completely abolished in *nst^{MZ}* mutants (Fig. 1B). Differentiation of dorsal mesoderm precursors, which is marked by expression of Even Skipped (Eve) and depends on Htl, was also affected in *nst^{MZ}* embryos (Fig. 1, C and D, and table S1). These genetic data indicated that *nst* function is required for FGF-dependent morphogenesis in the embryo.

Genetic interaction of *nst* with a hypomorphic allele of *htl* supported a role for Nst in Htl signaling. Whereas zygotic *nst* mutants (*nst^Z*) exhibited normal dorsal mesoderm differentiation, embryos homozygous for the hypomorphic *htl^{YY262}* allele showed a reduction in dorsal mesoderm (Fig. 1, C and D). Three independently generated alleles of *nst* zygotically enhanced the *htl^{YY262}* phenotype, indicating that *nst* is involved in FGF-dependent morphogenesis (Fig. 1, C and D, and table S1). Consistent with a role in Htl signaling, *nst* was required for Htl-dependent activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) in mesoderm cells (Fig. 1E), which is stimulated by the ectodermally produced FGF8-like ligands (15). MAPK activation downstream of other receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), was unimpaired in *nst^{MZ}* mutants (Fig. 1E and fig. S2). Thus, the *nst* mutation affected FGF-dependent, but not EGFR-dependent, signal transduction.

***nst* encodes PGM3 and is required for normal cellular levels of UDP-GlcNAc**

To determine the gene that is affected by *nst*, we mapped the original P element-induced mutation *nst¹⁶⁹²³* to the chromosomal interval 69C4-69D4-5 (fig. S3A). A lethal mutation within this interval, *pBacCG10627⁰⁴⁹⁸⁶*, inserted in the first intron of *CG10627* failed to complement both the lethality and the mesoderm phenotype of *nst¹⁶⁹²³* (fig. S3B). Sequencing genomic DNA of *nst¹⁶⁹²³* detected an 8-base pair duplication in *CG10627*, which leads to a frameshift producing a premature Stop codon after Asn²¹⁸. *CG10627* encodes the single fly homolog of human PGM3, and the predicted Nst¹⁶⁹²³ protein lacks three of four essential amino acid motifs of phosphohexomutases (fig. S3C) (16). To confirm that *nst* encodes PGM3, we created a transgene to express *CG10627* under the control of the *hsp70* promoter. Expression of *phsp70::CG10627* rescued the mesoderm and the tracheal phenotype, as well as the lethality of *nst^{MZ}* mutants (fig. S3, D to I, and table S2). We conclude that *nst* is allelic to *CG10627*.

Because PGM3 is an essential enzyme in the biosynthesis of UDP-GlcNAc (fig. S1), a crucial metabolite for cellular glycosylation, we were surprised that maternal null mutants of *nst* produced any embryos at all. Therefore, we measured the amount of cytoplasmic UDP-GlcNAc in wild-type and *nst^{MZ}* embryos by determining the amount of UDP-HexNAc using liquid chromatography–electrospray ionization tandem mass spectrometry (17). The amount of UDP-HexNAc in *nst^{MZ}* was only 17% of the amount determined in wild-type embryos, and UDP-HexNAc levels are restored to 80% upon expression of Nst from the rescue construct (Fig. 2A and table S3).

The rescue experiment suggested that the cytoplasmic UDP-GlcNAc was limiting for FGF signal transduction. We performed genetic analyses of another essential enzyme in the HBP UDP-GlcNAc-pyrophosphorylase, encoded by the *Drosophila mummy* (*mmy*) gene, to confirm this conclusion. Mmy converts the product of Nst, GlcNAc-1-phosphate, into UDP-GlcNAc (fig. S1). *mmy* mutants are zygotic lethal and do not produce embryos upon removal of the maternal expression. This phenotypic profile is consistent with an essential role for Mmy in UDP-GlcNAc biosynthesis (18–20). The amount of UDP-HexNAc in zygotic *mmy* mutants was ~50% of that of wild type and continuously dropped during further development (Fig. 2B and table S3). In contrast to *nst^{MZ}* mutants, however, embryos zygotically mutant for *mmy* did not exhibit any FGF signaling defects, suggesting that

~50% of the wild-type amount of UDP-GlcNAc was sufficient to support FGF signaling (Fig. 2, C and D). This difference in the biochemical and developmental phenotypes of *mmy* versus *nst* showed that Mmy was indispensable for the synthesis of UDP-GlcNAc, whereas the production of GlcNAc-1-phosphate occurred to some extent in the absence of *nst* during oogenesis. Thus, the loss of PGM3 activity in *nst* mutants resulted in a critical reduction of the cellular concentration of UDP-HexNAc that was unable to support normal FGF signaling.

Global protein O-GlcNAcylation is reduced in *nst*^{MZ} mutants

UDP-GlcNAc is a central metabolite for all N- and O-linked glycosylation, protein O-GlcNAcylation, GPI anchor formation, and proteoglycan biosynthesis. Because heparan sulfate proteoglycans (HSPGs) are necessary for FGF signaling (15), it seemed likely that UDP-GlcNAc deficiency would affect HSPG-dependent cell signaling. Instead, we found that *nst*^{MZ} mutants did not exhibit defects in other known HSPG-sensitive pathways, such as Wg and Hh signaling. We examined expression of *wg* and of its transcriptional target *engrailed* (*en*), which in turn acts through Hh to maintain *wg* expression. Immunohistochemistry for the Wg and En proteins indicated that *wg* expression and *en* expression were not affected in *nst*^{MZ} mutants, indicating that HSPG function was largely unimpaired by the UDP-GlcNAc deficiency (Fig. 2E). To test for GPI anchor formation, we ubiquitously expressed a GPI-green fluorescent protein (GFP) construct in *nst*^{MZ} embryos (Fig. 2F) and observed appropriate membrane association of the GPI-GFP fusion in the mutant embryos, indicating that the biosynthesis of GPI-anchored proteins was normal. We probed for global changes in N- and O-glycosylation using the lectins concanavalin A (Con A; detecting N-glycans at α -D-mannosyl and α -D-glucosyl residues) and jacalin (Jac; detecting O-glycans at Gal β 1-3GalNAc residues) to compare the pattern of abundant glycosylated proteins from embryo extracts (Fig. 2G). The protein patterns detected with Con A or Jac in *nst*^{MZ} mutants were similar to those detected in wild type. Thus, the UDP-GlcNAc deficiency in the *nst*^{MZ} mutants did not generally impair N- and O-glycosylation of abundant proteins.

Using a monoclonal antibody (RL-2) that recognizes terminal O-GlcNAc on proteins, we also compared cellular O-GlcNAc abundance in *nst*^{MZ} and wild-type embryos (21). This antibody labeled nuclear and cytoplasmic epitopes in embryos during germ band elongation with a slightly increased staining at the cephalic furrow and the neuro-ectoderm; the overall RL-2 immunoreactivity was reduced in *nst*^{MZ} embryos (fig. S4). On Western blots, this antibody recognized a characteristic pattern of O-GlcNAcylated proteins in embryos (Fig. 2H) and *Drosophila* S2 cells (fig. S5). We noted some differences in this pattern and that the overall amount of O-GlcNAcylation was reduced in *nst*^{MZ} embryos (Fig. 2H). We conclude that within the limits of detection of the methods used, *nst* is critical for global O-GlcNAcylation.

Nst acts downstream of FGFR signaling

The *nst* mutation caused a UDP-HexNAc deficiency that affected cytoplasmic protein O-GlcNAcylation, which could alter secretion and delivery of the FGF8-like ligands from the ectoderm or affect Htl signaling within the mesoderm cells or Btl signaling in the developing tracheal cells. To discriminate between these two possibilities, we determined the tissue-specific requirement of *nst*. If *nst* was required in the signal-receiving cells, a wild-type copy of Nst should be sufficient to rescue *nst*^{MZ} mutants in the cells expressing the FGFR. We expressed *UAS::Nst-HA* or a catalytically inactive mutant Nst[S68A] either in the mesoderm, using *twi::Gal4*, or in the developing trachea, using *btl::Gal4*. This targeted expression of wild-type Nst suppressed the mesoderm or the tracheal defects,

whereas the mutase-dead mutant form did not (Fig. 3, A to C, and table S4). Thus, the function of Nst in the signal-receiving cells alone is sufficient to sustain FGF signaling.

Because we determined that *nst* was required in the signal-receiving cells, we examined the epistasis of *nst* and *htl* by expressing a constitutively active receptor in which the extracellular domain is replaced by the dimerization domain of the λ Repressor (λ Htl) (22). Expression of λ Htl in the mesoderm rescues *htl* loss-of-function mutants as previously reported (22), and we found that in wild type or in *nst* heterozygotes, λ Htl produced a gain-of-function phenotype leading to an expansion of dorsal cell fates in the mesoderm (Fig. 3D). However, λ Htl expressed in the *nst*^{MZ} mutants failed to rescue either mesoderm differentiation or MAPK activation, indicating that Nst function was essential for signaling downstream of the receptor (Fig. 3, D to F, and table S5).

Reduced O-GlcNAcylation causes defects in FGF signaling

Because *nst* mutants were deficient in UDP-GlcNAc, exhibited reduced protein O-GlcNAcylation, and showed impaired FGF signaling downstream of the receptor, we hypothesized that FGF signaling required protein O-GlcNAcylation. To test whether reduced protein O-GlcNAcylation caused the FGF phenotype in *nst* mutants, we used the compound GlcNAcstatin C, which is a potent, selective, and cell-permeable small-molecule inhibitor of OGA that promotes O-GlcNAcylation when applied to cells in the nanomolar range (23, 24). Treatment of *Drosophila* S2 cells with GlcNAcstatin C enhanced protein O-GlcNAcylation (fig. S5C). Injection of GlcNAcstatin C into *nst*^{MZ} embryos suppressed the mesoderm defect and significantly increased the number of Eve-positive dorsal mesoderm precursors compared to control injected embryos (Fig. 4A and table S6). Similarly, increasing OGT activity in the cells by overexpressing OGT enhanced protein O-GlcNAcylation (fig. S5) and partially suppressed the mesoderm phenotype in *nst*^{MZ} embryos (Fig. 4B and table S7). Thus, the impaired FGF signaling in *nst* mutants was partially rescued by treatments that increase protein O-GlcNAcylation, indicating that the defects in FGF signaling in *nst* mutants are caused by impaired protein O-GlcNAcylation.

Loss-of-function mutations in OGT, encoded by the *super sex combs* (*sxc*) gene, do not affect embryonic mesoderm development but produce homeotic transformations in adults caused by the requirement of *sxc* for O-GlcNAcylation of the homeotic gene product Polyhomeotic (7, 9). We also did not observe a mesoderm defect in embryos zygotically mutant for both *sxc* and *nst* (fig. S6). Thus, similar to HBP enzymes, the maternal expression of *sxc* masks any functions of the gene during embryogenesis in zygotic mutants (25).

Dof is the critical component in MAPK activation affected in *nst* mutants

A distinct feature of the *nst*^{MZ} phenotype is its specificity for the FGF pathway. In the early embryo, MAPK (encoded by the *rolled* gene) is activated in a precisely controlled temporal-spatial pattern downstream of the receptor tyrosine kinases Torso, EGFR, and the FGFRs Htl and Btl (26, 27). In *nst* mutants, Torso and EGFR-dependent MAPK activation was normal, whereas FGFR-dependent MAPK activation was selectively reduced (Fig. 1E and fig. S2). The FGFR adaptor protein Downstream of FGFR (Dof) is specifically required for MAPK activation downstream of FGFRs, but not downstream of EGFR or Torso (22, 28, 29). Dof is also required for morphogenetic movements triggered by Htl in a pathway that is independent of MAPK activation (30). Because *nst* affected both Dof-dependent processes, we postulated that the *nst* phenotypes may result from a defect in Dof function.

To test this model, we bypassed the requirement for Dof by expressing a chimeric receptor, in which the Htl extracellular domain was fused to the tyrosine kinase domain of the Torso

receptor (Htl-Tor). These chimeric receptors signal upon activation by FGF ligands, but do not require Dof for signaling and thus suppress aspects of the phenotypes of *htl*, *btl*, or *dof* mutant embryos (31). Expression of Htl-Tor in the mesoderm of *nst*^{MZ} mutant embryos activated MAPK in the mesoderm, whereas a Btl-Htl chimeric receptor failed to activate MAPK (Fig. 4C). Thus, the function of the extracellular domain of the Htl receptor was unaffected in the UDP-HexNAc-deficient *nst* mutants, and MAPK activation in *nst* mutants was restored once the requirement for Dof was removed.

The FGFR adaptor protein Dof is glycosylated in an OGT-dependent fashion

To determine whether Dof was O-GlcNAcylated, we expressed Dof in S2 cells because Dof is present in low abundance in embryos. We used succinylated wheat germ agglutinin (sWGA) to enrich for proteins containing terminal GlcNAc conjugates characteristic of O-GlcNAcylated proteins (32). sWGA precipitated Dof from S2 cells, and this interaction was abolished by pre-absorption of sWGA with 0.5 M GlcNAc (Fig. 5A). Depletion of OGT in S2 cells by RNA interference (RNAi) reduced overall O-GlcNAcylation (fig. S5A), as well as sWGA-mediated precipitation of Dof (Fig. 5B). Conversely, overexpression of OGT in S2 cells promoted O-GlcNAcylation of cellular proteins (fig. S5B) and increased the amount of Dof in the sWGA-bound fraction (Fig. 5C). Blocking OGA with GlcNAcstatin C increased protein O-GlcNAcylation (fig. S5C) and increased sWGA-bound Dof in S2 cell lysates (Fig. 5D).

The data presented thus far do not exclude the possibility that binding of Dof to sWGA might be indirect and that Dof might be in a complex with other proteins that are O-GlcNAcylated. Therefore, we immunoprecipitated FLAG-tagged Dof and examined O-GlcNAc using sWGA on an Eastern blot. We detected Dof in the anti-FLAG immunoprecipitates, in particular in cells overexpressing OGT (Fig. 5E). These biochemical studies indicate that Dof is O-GlcNAcylated in an OGT-dependent way and support the model that Dof represents an essential substrate for OGT in the FGF signal transduction pathway (Fig. 6).

DISCUSSION

Protein O-GlcNAcylation represents the only known type of UDP-GlcNAc-dependent glycosylation of nuclear and cytoplasmic proteins, and its role in cell signaling networks is becoming increasingly recognized (33). This work demonstrated that protein O-GlcNAcylation is required for MAPK activation and morphogenetic movements downstream of FGFRs. The UDP-HexNAc deficiency caused by loss of *nst* was specific for FGF-dependent cell signaling. It is possible that the UDP-HexNAc deficiency might affect FGF signaling through negative effects on endomembrane-bound glycan formation. For example, the FGF ligands or the FGFRs might be specifically sensitive to changes in N-glycosylation. Our genetic in vivo data argue against this possibility. First, we excluded a requirement for glycosylation of the FGF8-like ligands because we demonstrated that *Nst* function in the signal-receiving cells alone was sufficient for signaling. Furthermore, activation of the FGF pathway with λ Htl neither activated MAPK nor mediated dorsal mesoderm differentiation in UDP-HexNAc-deficient embryos. Therefore, irrespective of a potential defect in the glycosylation of the FGFR, all aspects of the *nst* FGF-specific phenotype were still present even when the pathway should be activated in a ligand-independent fashion. Finally, we demonstrated that ligand-dependent dimerization of a chimeric FGFR activated MAPK in UDP-HexNAc-deficient embryos.

We determined that the UDP-GlcNAc sensitivity of this FGF-specific signaling event was upstream of the canonical Ras to Raf MAPK cassette. The adaptor protein Dof is the only known molecule that discriminates MAPK activation by FGFR signaling from that by other

receptor tyrosine kinases in *Drosophila* (22, 28, 29). Moreover, *nst* mutations also affected MAPK-independent responses of the FGF signaling pathway, such as mesoderm layer formation (32, 33). Together with our data showing that Dof is O-GlcNAcylated, this suggests that O-GlcNAcylation of Dof is essential for its function as an adaptor-scaffold in FGF signaling (Fig. 6). Although our data are also consistent with the intracellular domain of the FGFR as a target for O-GlcNAcylation, we did not detect O-GlcNAc modification of the receptor. Therefore, Dof provides a node for both pathways, the HBP and the FGF pathway, and thus, a defect in its posttranslational modification appears to be responsible for all of the phenotypes associated with deficient FGF signaling observed in *nst*^{MZ} embryos (Fig. 6).

Increasing protein O-GlcNAcylation by genetic and pharmacological manipulation of OGT and OGA activity suppressed the *nst*^{MZ} phenotype. The limited suppression of the phenotype by blocking OGA activity might be due to the low amount of UDP-GlcNAc in the mutant and the reversible nature of the O-GlcNAc modification on proteins, called O-GlcNAc cycling. In the case of OGT overexpression, increased O-GlcNAc cycling will further decrease cytoplasmic UDP-GlcNAc concentrations in the absence of Nst. During O-GlcNAc hydrolysis, OGA produces GlcNAc, which can be phosphorylated by GlcNAc kinase to produce GlcNAc-6-phosphate. GlcNAc-6-phosphate can enter the HBP and lead to the production of UDP-GlcNAc, but this recovery pathway is dependent on Nst. Therefore, increased O-GlcNAc cycling in *nst* mutants is expected to deplete cytoplasmic UDP-GlcNAc.

The results described here provide evidence for a critical signaling node that connects the control of cell growth, survival, and morphogenesis by FGF signaling with a posttranslational modification (O-GlcNAcylation) that is regulated by flux through a metabolic pathway (the HBP) that integrates the availability of nutrients (glucose and glutamine) and energy-dependent metabolites. It will be interesting to see whether this signaling node transmits metabolic signals by modulating FGF signaling responses.

MATERIALS AND METHODS

Drosophila genetics

The following stocks were used: *w*¹¹¹⁸, *Df(3L)4486/TM6*, *pBacCG10627⁰⁴⁹⁸⁶*, *w;nst¹⁶⁹²³FRT2A/TM3[ftz::lacZ]*, *w;P[ovo^{D1-18}]FRT2A/βtub^{85D}/TM3, twi::GAL4*, *htl^{YY262}/TM3[ftz::lacZ]*, *UAS::λhtl*, *wg^{CE7}/CyO[ftz::lacZ]*, *mmy^{JK63}/CyO[ftz::lacZ]*, *btl^{dev1}/CyO[ftz::lacZ]*, *btl::GAL4/CyO*, *UAS::htl*, *UAS::btl-htl*, *sxc¹/CyO*, *sxc⁶/CyO*, and *actin::GAL4*, *UAS::GPI-eGFP*. The screen for maternal effect loci was performed as outlined in (34). Microinjections were performed on dechorionated syncytial-stage embryos with 0.1 M GlcNAcstatin C (GlycoBioChem) or dimethyl sulfoxide (DMSO) as control. After being aged at 18°C in a humidified chamber overnight, the embryos were fixed and immunostained.

Antibodies and immunostaining

Embryos were fixed and immunolabeled as described (35). Microscopy was performed with an Olympus BX61 attached to a Volocity grid confocal system (Improvision) and a Leica SP2 confocal microscope. Images were processed with Adobe Photoshop and Volocity (Improvision). Secondary antibodies were obtained from Jackson ImmunoResearch (Strattec). For immunostaining, the following antibodies were used: rabbit antibody recognizing β-galactosidase (βGal, 1:5000, Cappel), mouse antibody recognizing βGal (1:100), mouse antibody recognizing Eve (1:80), mouse antibody recognizing O-GlcNAc (RL-2, ABCAM) (1:100), mouse antibody recognizing Wg(1:40), mouse antibody

recognizing En (1:10) [all mouse monoclonal antibodies, except RL-2, were obtained from Developmental Studies Hybridoma Bank (DSHB)], rabbit antibody recognizing Twi (1:1000)(36), mouse antibody recognizing dpERK (Sigma-Aldrich), and rabbit antibody recognizing Verm (1:300; a gift from S. Luschnig).

Western blotting

Lysates were prepared and analyzed by Western blot as described (37). The following antibodies were used: mouse antibody recognizing *O*-GlcNAc (RL2) (1:1000, Abcam), mouse antibody recognizing FLAG (1:1000, Sigma), and mouse antibody recognizing α -tubulin (1:1000, DSHB). For Eastern blots, biotin-conjugated Con A (1:3000), Jac (1:2000), or succinylated wheat germ agglutinin (1:250) was used in combination with ExtrAvidin horseradish peroxidase (HRP) (1:10,000) (Sigma-Aldrich). The specificity of the binding was controlled by preincubation of the RL-2 antibody with 0.5 M GlcNAc, preincubation of Con A with 0.5 M α -methylmannopyranoside, or preincubation of Jac with 0.8 M galactose.

Sugar nucleotide analysis

Sugar nucleotide analysis was performed as described elsewhere (17). Briefly, 40 to 45 embryos were lysed in ice-cold phosphate-buffered saline and extracted with 70% ethanol in the presence of 20 pmol of guanosine diphosphate–glucose internal standard (a sugar nucleotide that does not occur in *Drosophila* embryos). Sugar nucleotides were extracted by means of ENVI-Carb columns and analyzed using multiple reaction–monitoring liquid chromatography–tandem mass spectrometry (17). Protein amount was determined with Bradford reagent (Fermentas).

Molecular biology

Molecular cloning and germline transformation were performed using standard procedures. Primer sequences are provided in table S8. pUAST-HA vector was used to generate transgenic lines containing the coding sequences for Nst, Nst[S68A], OGA, or OGT. The rescue construct contained a genomic region of Nst cloned into pCasper4.

Cell culture, transfection, and immunoprecipitation

Drosophila S2 cells were cultured in Schneider's medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin (Gibco). Cells plated at 1×10^6 were allowed to adhere for 1 hour. Transfections were carried out by mixing FuGENE HD (Roche):DNA (2 μ g) at the ratio of 3:2 (μ l: μ g) in 100 μ l of sterile water. The constructs used for transfections were pMT-GAL4, pRmHa-FLAG-Dof, and pUAS-OGT-HA. The metallothionein promoter was induced with 1 mM CuSO₄ 24 hours after transfection. Where indicated, GlcNAcstatin C was added to cell culture medium at a concentration of 1 μ M. Cells were lysed with nondenaturing lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton, 1 μ M GlcNAcstatin C, 1 mM sodium orthovanadate, 5 mM sodium fluoride, and protease inhibitors]. RNAi was performed 48 hours before DNA transfections by transfecting 4 μ g of double-stranded RNA (dsRNA) directed against the appropriate transcript. Immunoprecipitation of FLAG-Dof was performed with protein G–coupled Dynabeads (Invitrogen). Lysates were incubated overnight with 30 μ l of sWGA-conjugated agarose beads (Vector Labs) prewashed with lysis buffer. The immunoprecipitates, after removal of the unbound fraction, were washed with lysis buffer and processed for immunoblot analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

nst mutant phenotype and genetic interaction with *htl*. (A) Cross sections of embryos of the indicated genotypes at the extended germ band stage (stage 9). Antibodies recognizing Twi (black) mark mesoderm cell nuclei. WT, wild type. (B) Trachea of stage 15 embryos of the indicated genotypes stained with antibodies recognizing Verm; the dorsal trunk (DT) is marked by arrows. (C) Dorsal mesoderm cells of stage 11 embryos of the indicated genotypes stained with antibodies against Eve (arrowheads). (D) Quantification of Eve clusters for genotypes depicted in (C). Bars with the same symbol show values that are significantly different ($P < 0.01$; $n = 35$ embryos; table S1). (E) Stage 8 embryos stained with antibodies against Twi (green) and antibodies against dpERK (red); merged images to the right. Top row, WT embryos; bottom, *nst^{MZ}* embryos. Arrows indicate Htl-dependent MAPK activation in mesoderm. Arrowheads indicate EGFR-dependent MAPK activation in *nst^{MZ}* embryos (see also fig. S2). *htl*: *htl^{AB42}* zygotic homozygote; *nst^{MZ}*: *nst¹⁶⁹²³* maternal zygotic mutants; *nst^Z*: *nst¹⁶⁹²³* zygotic homozygotes; *htl^{YY}*: *htl^{YY262}* zygotic homozygotes; *htl^{YY}, nst^Z*: *htl^{YY262}, nst¹⁶⁹²³* zygotic double mutants; *nst^{Df}*: *Df(3L)4486* homozygotes.

Fig. 2.

UDP-HexNAc nucleotide biosynthesis and glycosylation in *nst* mutants. **(A)** Relative amount of UDP-HexNAc in embryo (stages 9 to 11) extracts of the indicated genotypes ($n = 2$ experiments at 40 to 45 embryos per sample; table S3). **(B)** Relative amount of UDP-HexNAc in *mmy* and *nst^{MZ}* mutants ($n = 2$ experiments; table S3). **(C)** Eve staining of dorsal mesoderm in stage 11 embryos of the indicated genotypes. **(D)** Verm staining of the trachea in stage 15 embryos of the indicated genotypes. **(E)** Wg signaling was detected in stage 10 embryos of the indicated genotypes with antibodies recognizing Wg (left) or En (right). **(F)** GPI-anchored GFP (gpi-GFP) in the indicated *nst* embryos. Scale bar, 5 μ m. **(G)** Eastern blot of protein lysates of stage 9 to 11 WT and *nst^{MZ}* embryos with concanavalin A (Con A) or jacalin (Jac). Where indicated, lectins were preincubated with either 0.5 M α -methylmannopyranoside (MMP) for Con A or 0.8 M galactose (Gal) for Jac. A nonspecific band reactive with ExtrAvidin HRP (2°) was used as loading control (arrow). Black lines indicate separation of differently treated nitrocellulose filters originating from the same SDS-polyacrylamide gel electrophoresis (SDS-PAGE). **(H)** Western blot of protein lysates from stage 9 to 11 WT or *nst^{MZ}* embryos probed with antibodies recognizing O-GlcNAc

(anti-*O*-GlcNAc monoclonal antibody RL-2). Where indicated, antibodies were preincubated with 0.5 M GlcNAc. α -Tubulin (α tub) served as loading control. Data in (G) and (H) are representative of three experiments. *nst^Z*, zygotic *nst^{I6923}* homozygotes; *nst^{MZ}* *hs>CG10627*, *nst^{I6923}* maternal zygotic mutants expressing the rescue construct *phsp70::CG10627*; *mmy^Z*, zygotic *mmy^{IK63}* homozygotes; *wg*, *wg^{CE7}* homozygotes; *nst^M*, *nst^{I6923}* maternal mutant with WT *nst* gene expressed from paternal chromosome. Molecular masses are indicated in kilodaltons.

Fig. 3.

Epistatic relationship between *nst* and *htl*. (A) Expression of *UAS::Nst* rescues *nst*^{MZ} mutant cells autonomously. Embryos of the indicated genotypes were stained with antibodies recognizing either Eve (stage 11, top row) or Verm (stage 15, bottom row). (B) Quantification of Eve-positive hemisegments in embryos of indicated genotypes ($n = 35$ or 50 embryos; table S4). (C) Percentage of embryos with either intact dorsal trunk (DT) or the presence (Verm+) or absence (Verm-) of Verm staining ($n = 100$ embryos) for the indicated genotypes. The mutase-dead *Nst*[S68A] mutant served as a control. (D) Expression of constitutively active Htl (λ Htl) in the mesoderm of *nst* mutant embryos stained with antibodies recognizing Eve. Arrows mark enlarged Eve cell clusters caused by expression of λ Htl in maternal *nst* (*nst*^M) mutants, which are lacking in the *nst*^{MZ} mutants expressing λ Htl. (E) Quantification of the Eve-positive hemisegments of embryos expressing λ Htl in *nst*^{MZ} mutants ($n = 35$ embryos; see table S5). (F) MAPK activation in the mesoderm was detected with antibodies against dpERK (red) in stage 8 embryos with λ Htl in maternal *nst*^M mutants (top row) and *nst*^{MZ} mutants expressing λ Htl (bottom row). The mesoderm nuclei were stained with antibodies recognizing Twi (green) and the presence of the paternal balancer chromosome by anti- β Gal (green). *twi*>>*Nst*: *twi*::*Gal4*, *UAS::Nst*; *btl*>>*Nst*:

*btl::Gal4,UAS::Nst; twi>>NstS68A: twi::Gal4,UAS::Nst[S86A]; btl>>NstS68A: btl::Gal4,
UAS:: Nst[S86A]; twi>>λHtl: twi::Gal4,UAS::λHtl.*

Fig. 4.

FGF signaling in *nst* embryos upon altered protein O-GlcNAcylation. (A) Embryos of the indicated genotypes were injected at syncytial stages with GlcNAcstatin C (GC) or DMSO (DM) as a control, fixed at stage 11, and stained with antibodies against Eve. Eve-positive hemisegments were quantified for the indicated genotypes and injection protocols ($n = 10$ to 19 embryos; table S6). (B) Quantification of Eve-positive hemisegments in *nst^{MZ}* embryos overexpressing *UAS::OGT* or *UAS::OGA* in the mesoderm ($n = 35$ embryos; table S7). (C) Activation of MAPK in response to the expression of chimeric receptor tyrosine kinases in the mesoderm of *nst^{MZ}* embryos was detected in fixed stage 8 embryos immunolabeled with Twi (green) and dpERK (red) antibodies. Arrows mark *nst*-independent activation of MAPK in the ectoderm, and arrowheads mark MAPK activation in the mesoderm (positive for Twi). *btl-htl: UAS::btl-htl*, fusion of the extracellular domain of the Btl FGFR and the intracellular domain of Htl; *htl-tor: UAS::htl-tor*, fusion of the extracellular domain of Htl and the cytoplasmic domain of Tor; *twi>>OGT: twi::Gal4,UAS::OGT*; *twi>>OGA: twi::Gal4,UAS::OGA*.

Fig. 5. OGT-dependent glycosylation of Dof. Lysates of FLAG-Dof-expressing S2 cells were subjected to sWGA affinity binding (IP: sWGA) followed by immunoblotting with antibodies recognizing the FLAG epitope (WB: anti-FLAG) (**A** to **D**) or were subjected to immunoprecipitation using antibody against FLAG (IP: anti-FLAG) followed by Eastern blot using sWGA (EB: sWGA) (**E**). Input lanes show lysates of cells with and without transfection of FLAG-Dof and arrowheads mark full-length FLAG-Dof. (**A**) Precipitation of FLAG-Dof from S2 cell lysate by sWGA beads. GlcNAc: control of FLAG-Dof binding to sWGA by preabsorbing sWGA beads with 250 mM GlcNAc. (**B**) Effect of RNAi (dsRNA) against OGT on FLAG-Dof binding to sWGA. Cells treated with dsRNA directed against OGT or GFP (control). (**C**) Coexpression of FLAG-Dof with OGT-HA. Global protein O-GlcNAcylation in S2 cells upon expression of OGT-HA is depicted in fig. S5B. (**D**) Effect of GlcNAcstatin C on FLAG-Dof O-GlcNAcylation. S2 cells overexpressing FLAG-Dof were treated with 1 μ M GlcNAcstatin C or DMSO as control. Global protein O-GlcNAcylation in S2 cells after treatment with GlcNAcstatin C is depicted in fig. S5C. (**E**) Anti-FLAG immunoprecipitates from lysates of FLAG-Dof-expressing S2 cells analyzed by Eastern blotting (EB) using sWGA. Molecular masses are indicated in kilodaltons. Data are representative of three separate experiments.

Fig. 6.

Model for O-GlcNAcylation in FGF signaling. The model shows the requirement of protein O-GlcNAcylation for Htt-dependent mesoderm spreading mediated by the Pebble (Pbl)–Rac pathway and mesoderm differentiation mediated by MAPK activation. The HBP converts glucose (blue circle) into UDP-GlcNAc (blue squares). With normal amounts of UDP-GlcNAc, OGT transfers O-GlcNAc to the adaptor protein Dof. O-GlcNAcylated Dof may be phosphorylated (red circles) upon activation of Htt by FGF8-like ligands and recruits effector proteins, such as the phosphatase Corkscrew (Csw), to trigger FGF-dependent MAPK activation and cell shape changes. This model does not exclude the possibility that OGT might also O-GlcNAcylate yet unknown protein(s) (X), which may be indirectly required for FGF signaling (transparent brown arrow).