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Wollknäuel is required for embryo patterning and encodes the *Drosophila* ALG5 UDP-glucose:dolichyl-phosphate glucosyltransferase

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N-linked glycosylation is a prevalent protein modification in eukaryotic cells. Although glycosylation plays an important role in cell signaling during development, a role for N-linked glycosylation in embryonic patterning has not previously been described. In a screen for maternal factors involved in embryo patterning, we isolated mutations in *Drosophila* ALG5, a UDP-glucose:dolichyl-phosphate glucosyltransferase. Based on the embryonic cuticle phenotype, we designated the ALG5 locus *wollknäuel* (*wol*). Mutations in *wol* result in posterior segmentation phenotypes, reduced Dpp signaling, as well as impaired mesoderm invagination and germband elongation at gastrulation. The segmentation phenotype can be attributed to a post-transcriptional effect on expression of the transcription factor Caudal, whereas *wol* acts upstream of Dpp signaling by regulating *dpp* expression. The *wol/ALG5* cDNA was able to partially complement the hypoglycosylation phenotype of *alg5* mutant *S. cerevisiae*, whereas the two *wol* mutant alleles failed to complement. We show that reduced glycosylation in *wol* mutant embryos triggers endoplasmic reticulum stress and the unfolded protein response (UPR). As a result, phosphorylation of the translation factor eIF2α is increased. We propose a model in which translation of a few maternal mRNAs, including *caudal*, are particularly sensitive to increased eIF2α phosphorylation. According to this view, inappropriate UPR activation can cause specific patterning defects during embryo development.

KEY WORDS: *Drosophila*, Glycosylation, Patterning, Unfolded protein response

INTRODUCTION

A common protein modification in eukaryotic cells is N-linked glycosylation, which occurs on the majority of proteins synthesized in the endoplasmic reticulum (ER), where a pre-assembled oligosaccharide chain is transferred to the nascent polypeptide (Helenius and Aebi, 2004). Protein glycosylation has several purposes: it is needed for proper folding and quality control in the ER, it targets some proteins to different cellular compartments, and it can affect protein function. Accumulation of unfolded proteins within the ER triggers the unfolded protein response (UPR) (Zhang and Kaufman, 2004). This response increases the folding capacity of the ER and decreases the folding demand via three ER transmembrane proteins, IRE1 (inositol-requiring 1), ATF6 (activating transcription factor 6) and PERK (PKR-like endoplasmic reticulum kinase). Activation of IRE1 and ATF6 causes transcriptional activation of genes needed for folding in the ER, whereas PERK activation results in a general decrease in translation initiation and selective translation of specific mRNAs through phosphorylation of eIF2α.

Transfer of Met-tRNA$_i$ to the 40S ribosomal subunit is accomplished by GTP-bound eIF2 (Proud, 2005). Following recognition of the AUG start codon, GTP is hydrolyzed and the eIF2-GDP complex released from the ribosome. Exchange of GDP for GTP is mediated by eIF2B, and is regulated by phosphorylation of the alpha subunit of eIF2 at a conserved serine residue, which generates a competitive inhibitor of eIF2B.

In this work, we show that mutations in *wollknäuel*, a UDP-glucose:dolichyl-phosphate glucosyltransferase involved in N-linked protein glycosylation, disrupts *Drosophila* embryo development by affecting the expression of a few key gene regulators. Reduced glycosylation efficiency in *wol* mutant embryos triggers the UPR. As a result, phosphorylation of eIF2α is increased. We propose that some mRNAs are more sensitive to eIF2α phosphorylation than others, and that this causes specific patterning defects.

MATERIALS AND METHODS

Fly stocks, P-element transformation and germline clones

Oregon-R or w^{1118} were used as wild-type controls. The *wol* alleles 2L-284 (wol^1) and 2L-267 (wol^2), as well as the *cad* allele 2L-264, were generated on an FRT$^{2L\text{-}40A}$-containing chromosome in a germline clone EMS screen performed in Tübingen (Luschnig et al., 2004).

A P-element plasmid containing a 2.8 kb *wol/ALG5* genomic region was constructed by PCR amplification from genomic DNA, cloned into pCaSpeR4 (Thummel and Pirrotta, 1992), and injected into w^{1118} embryos according to standard procedures. Two insertions on the X-chromosome were used in rescue experiments.

Germline clones were produced as described (Qi et al., 2008). Males containing a transgene misexpressing *dpp* in the *Krüppel* (*Kr*) domain (gift from Hilary Ashe, University of Manchester, UK) were crossed to wol^1 germline clone females. Expression of *dpp* was activated by FLPing out transcriptional stop signals downstream of the *Kr* promoter (Struhl et al., 1993).

Cuticle preparation, in situ hybridization and immunofluorescence

Cuticles were prepared as described by Wieschaus and Nüsslein-Volhard (Wieschaus and Nüsslein-Volhard, 1998) and examined using dark-field microscopy. Whole-mount RNA in situ hybridization using digoxigenin-

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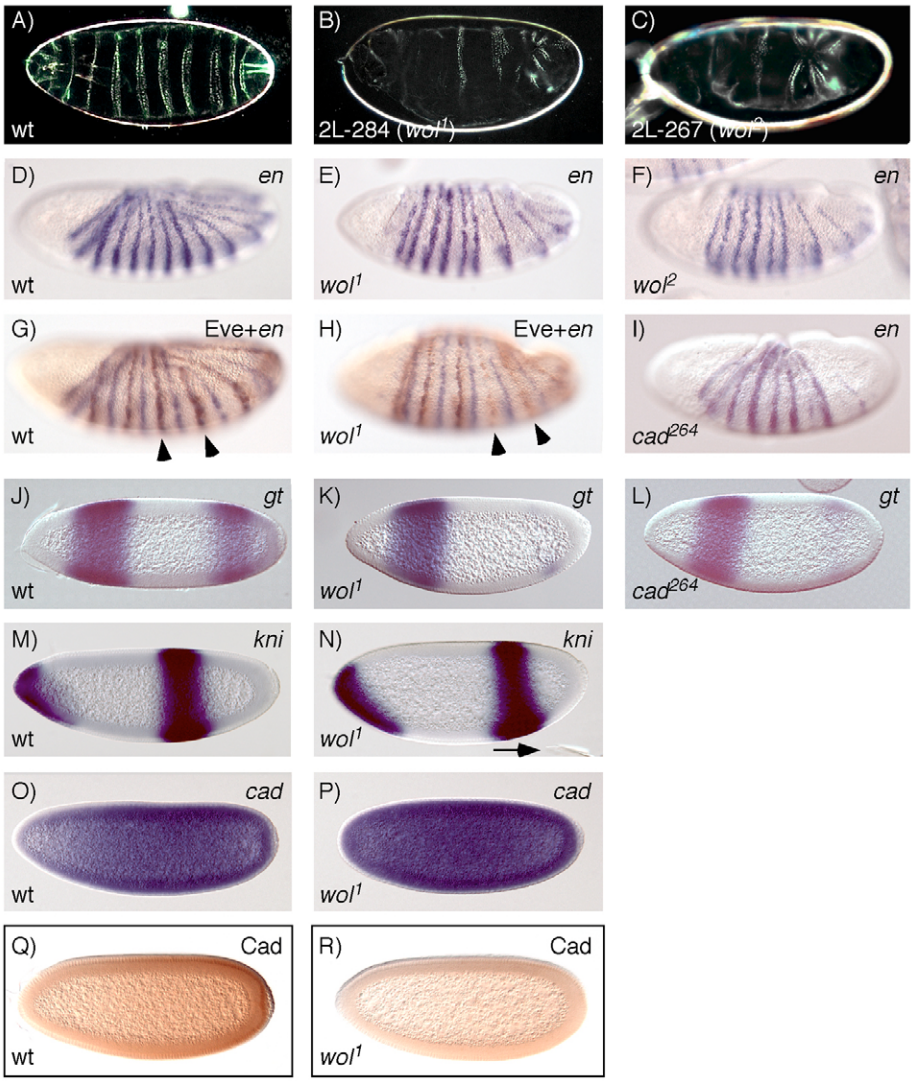

Fig. 1. Posterior patterning is disrupted in embryos derived from *wol* germline clones. Anterior is to the left in all figures. Genotypes, RNA probes and antigens are indicated. (**A-C**) Cuticle preparations of *Drosophila* embryos prior to hatching. Some posterior denticle belts are missing and the remainder are curled-up in *wol* embryos, as compared with wild type (wt). (**D-I**) Ventrolateral views of embryos undergoing germband elongation. Every other *en* stripe is missing in the posterior of *wol* embryos. (G,H) Eve protein (brown) labels the odd-numbered *en* stripes (blue). Arrowheads point to *en* stripes 7 and 9. (I) Posterior *en* stripes are missing in embryos from *cad* germline clones. (**J-N**) Lateral views of pre-cellular embryos showing changes in *gt* and *kni* expression in *wol* and *cad* embryos as compared with wild type. Arrow in N indicates a shift in position of the *kni* stripe towards posterior. (**O-R**) Maternal *cad* mRNA is present at similar levels in wild-type and *wol* mutant embryos, whereas the Cad protein gradient is reduced in *wol* embryos (R).

labeled probes was performed as described previously (Jiang et al., 1991; Tautz and Pfeifle, 1989). Double labeling was performed as described (Kosman and Small, 1997).

Immunohistochemistry and RNA/protein double-staining protocols are modified from Manoukian and Krause (Manoukian and Krause, 1992). Rabbit anti-Eve (1:1000 dilution) was provided by Mike Levine (University of California, Berkeley, CA), guinea pig anti-Cad (1:800) and rat anti-Hb (1:400) antibodies are described by Kosman et al. (Kosman et al., 1998) and were provided by John Reinitz and Steve Small.

Positional cloning

Complementation tests with deficiencies were used to map the *wol* alleles, which failed to complement the deficiency *Df(2L)TE29Aa-11*. We developed SNP markers from the intergenic regions in the 260 kb interval of this deficiency. PCR products that could be distinguished by restriction fragment length polymorphisms were identified, and used in high-resolution recombination mapping with P-element strains flanking the deficiency. The wol^1 allele in a $white^-$ background was crossed to the $white^+$ P-elements l(2)k16919, located proximal to the deficiency, or l(2)k14902, positioned distal to the deficiency. We recovered 25 white-eyed recombinants out of ~20,000 flies from the cross with the proximal P-element, and four recombinants out of ~5000 flies from the distal P-element. Genomic DNA was prepared from the recombinants, PCR amplified with SNP marker primers, and analyzed after restriction digest. One recombination event with the l(2)k16919 chromosome occurred distal to a SNP located 162 kb into the deficiency. Two out of the four recombinants with l(2)k14902 had recombined proximal to a SNP located 210 kb into the deficiency.

Genomic DNA was isolated from wol^1 and wol^2 homozygous mutant larvae. Exonic sequences from the genes in this interval were amplified by PCR, sequenced and compared with an $FRT^{2L\text{-}40A}$ chromosome from another mutant. We found mutations in the CG7870/*ALG5* gene, and confirmed that lethality maps to the *ALG5* locus by complementation tests with a PiggyBac insertion (PBac RB^{e04276}, Fig. 3) that became available during the course of this work.

Yeast assays

Saccharomyces cerevisiae strains used were derivates of YG91 (*Matα ade2-101 ura3-52 his3-200 Δalg5::HIS3*) and YG355 (*Mat ade2-101 ura3-52 his3-200 Δalg5::HIS3 wbp1-2*) (Burda et al., 1996). Standard yeast media and genetic techniques were used (Guthrie and Fink, 1991).

The *Drosophila* ALG5 cDNA was RT-PCR amplified from embryo mRNA, TA-cloned and sequenced. The wol^1 and wol^2 mutations were introduced by site-directed mutagenesis (Quick-change Kit, Stratagene). Blunt-ended cDNAs were cloned into the yeast expression vector pCFZ41-GPD (Mumberg et al., 1995).

Analysis of carboxypeptidase Y (CPY) in *ALG5*-deficient cells (YG91) was detected by western blot analysis as described (Burda et al., 1996).

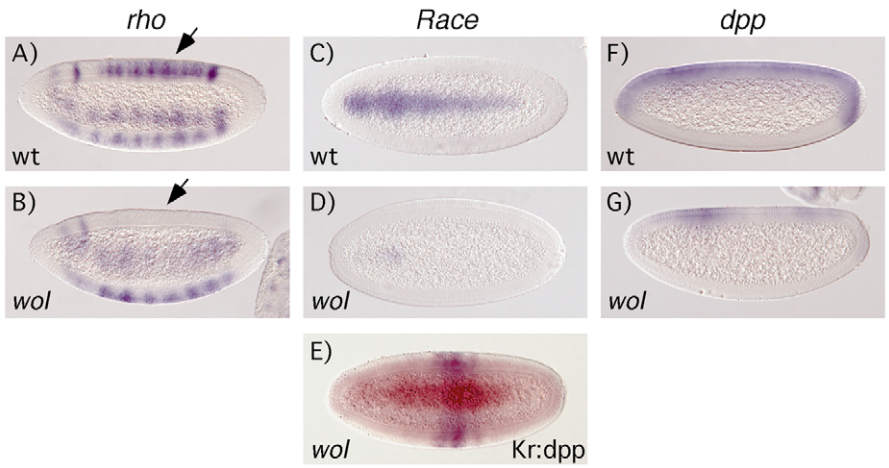

Fig. 2. Reduced Dpp signaling causes dorsal-ventral phenotypes in *wol*[1] germline clone embryos. (**A**,**B**) Ventrolateral views of cellularized *Drosophila* embryos. Expression of *rho* in dorsal cells occurs in wild type in response to Dpp signaling, but is lost in *wol* embryos (arrows). (**C-E**) Dorsal views of cellularized embryos showing expression of the Dpp target gene *Race* (C,D) or of *Race* and *dpp* (E). Ectopic *dpp* (purple) expressed from the *Krüppel* enhancer (Kr:dpp) was able to rescue *Race* expression (brown) in *wol* mutant embryos (E). (**F**,**G**) Lateral views of cellularizing embryos. Expression of *dpp* is reduced in *wol* embryos (G).

Western blot and RT-PCR

Protein extracts from 2×10^6 untreated, 10 mM DTT-treated or eIF2α RNAi-treated S2 cells, or from 30 μl 2- to 4-hour w^{1118} or wol^1 germline clone embryos were prepared as described (Lilja et al., 2007). Proteins (7 μg) were separated by SDS-PAGE, transferred to PVDF membrane (GE Healthcare), and incubated with a rabbit phospho-eIF2α (Ser51) antibody (1:1000, Cell Signaling Technology). The membrane was re-probed with a rabbit anti-human eIF2α (residues 50-150) antibody (1:200, Abcam). HRP-coupled secondary antibodies were visualized by ECL (GE Healthcare).

Total RNA was isolated from 0- to 3-hour w^{1118} and wol^1 germline clone embryos using TRIzol reagent (Invitrogen). PolyA-RNA was extracted from total embryo RNA as well as from mock-treated, DTT-treated and RNAi-treated cells using Dynabeads mRNA DIRECT Microkit (DYNAL), followed by reverse transcription with Superscript II (Invitrogen). Primers flanking the unconventional splice site in *xbp1* mRNA (Xbp1_F, 5′-CGCCAGCGCAGGCGCTGAGG-3′ and Xbp1_R, 5′-CTGCTCCGCC-AGCAGACGCGC-3′) were used in 25 PCR cycles. RNAi against *eIF2α* was performed as described (Qi et al., 2008).

RESULTS AND DISCUSSION

Segmentation defects in *wollknäuel* mutant embryos

From a screen for maternal genes that are required for *Drosophila* embryo development (Luschnig et al., 2004), we selected mutants that affect segmentation gene expression. We identified two mutants with defects in posterior embryo patterning, 2L-284 and 2L-267, that failed to complement each other and are thus allelic. Cuticle preparations of embryos derived from mothers harboring 2L-284 or 2L-267 germline clones revealed segmentation defects in the posterior half of the embryo, and a curled-up phenotype resulting from defects in germband elongation and retraction (Fig. 1B,C). The cuticle phenotype resembles a ball of wool, Wollknäuel in German, and we therefore named our alleles *wollknäuel* 1 and 2 (wol^1 and wol^2).

We stained embryos derived from *wol* germline clones (hereafter called *wol* embryos) with an *engrailed* (*en*) RNA probe (Fig. 1D-F). In the posterior, every second *en* stripe was missing. Double staining for Even skipped (Eve) protein and *en* RNA showed that the missing stripes correspond to odd-numbered stripes (Fig. 1G,H). Expression of *en* regulators was altered in *wol* embryos (see Fig. S1 in the supplementary material). We therefore examined gap gene expression, as gap proteins control expression of the *en* regulators. The posterior *giant* (*gt*) expression domain was severely reduced (Fig. 1K), although it recovered at later embryo stages (not shown), and the posterior *knirps* (*kni*) stripe was shifted posteriorly in *wol* embryos (Fig. 1, compare M with N).

A major activator of *gt* and *kni* expression is Caudal (Cad), which forms a posterior-anterior protein gradient, being translationally repressed by Bicoid in the anterior part of the embryo (Rivera-Pomar and Jackle, 1996). Whereas the maternal *cad* RNA was present in normal amounts in *wol* embryos (Fig. 1, compare O with P), we found much less Cad protein in *wol* embryos than in the wild type (Fig. 1Q,R). This result suggests that either Cad protein stability or the efficiency of Cad translation is affected by the *wol* mutations. Segmentation gene expression in *wol* embryos was very similar to that found in embryos derived from *cad* germline clones (Fig. 1I,L). There was no failure in either the terminal system or in the posterior system, both of which control gap gene expression (see Fig. S2 in the supplementary material). We therefore favor the notion that reduced Caudal levels cause the segmentation phenotype in *wol* embryos.

wol is required for dorsal-ventral patterning and gastrulation movements

We investigated dorsal-ventral patterning in *wol* embryos by examining *rhomboid* (*rho*) expression. The *rho* gene is expressed in two ventrolateral bands in response to the protein Dorsal (Stathopoulos and Levine, 2002). In addition, *rho* is activated in dorsal cells by signaling from the TGF-β protein Decapentaplegic (Dpp) (Fig. 2A, arrow). In *wol* mutant embryos, the Dpp-dependent *rho* expression pattern was selectively affected (Fig. 2B). We overexpressed Dpp from a *Kr* enhancer in a central domain of transgenic embryos and monitored Dpp activity through another downstream target gene, *Race* (also known as *Ance*) (Fig. 2C) (Rusch and Levine, 1997). As expected, *Race* mRNA was absent from *wol* mutant embryos (Fig. 2D). However, in *wol* embryos expressing ectopic Dpp (purple in Fig. 2E), *Race* expression was restored (brown in Fig. 2E). From this result, we conclude that *wol* activity is not needed for transduction of the Dpp signal, but is acting upstream of Dpp signaling. We found that expression of *dpp* itself is reduced in *wol* embryos as compared with wild type (Fig. 2F,G), which probably explains the failure to activate Dpp target genes. It appears that an unknown maternal regulator of *dpp* expression or, alternatively, mRNA stability is dependent on wild-type *wol* activity.

We also noted problems with cell movements during gastrulation in *wol* mutant embryos. As a result, germband elongation does not proceed normally (Fig. 1 and data not shown), and mesoderm invagination is disturbed (see Fig. S3 in the supplementary material).

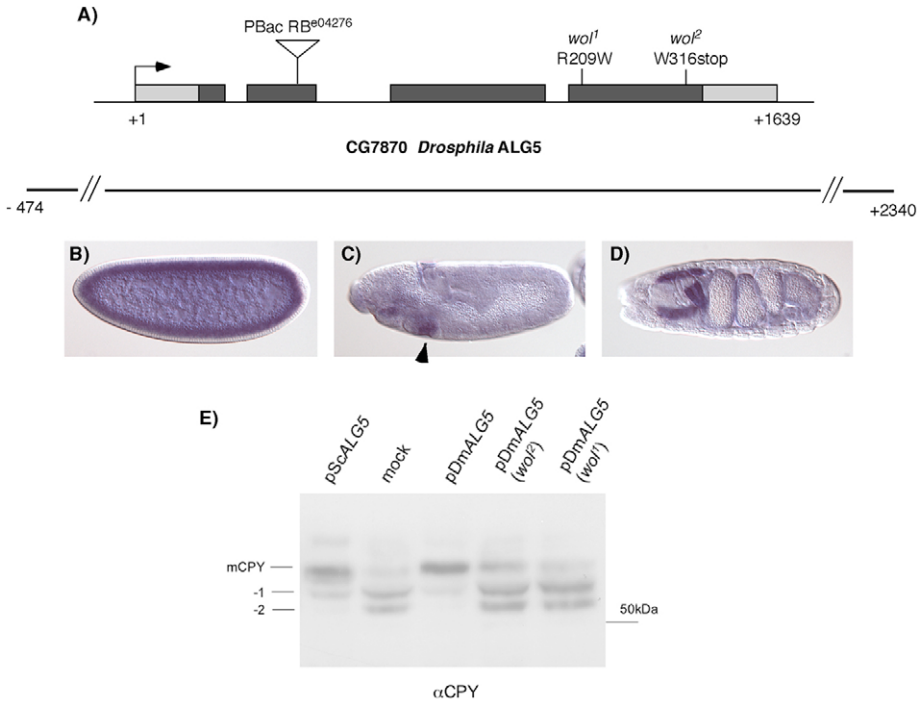

Fig. 3. *wol* encodes the *Drosophila* ALG5 UDP-glucose:dolichyl-phosphate glucosyltransferase. (**A**) The gene CG7870/*ALG5* consists of four exons encoding a 326 amino acid protein (coding region shaded dark gray). The lesions in the wol^1 and wol^2 alleles are shown. A transgene extending from –474 to +2340 was able to rescue the lethality and fertility of the *wol* alleles. (**B-D**) Expression of *wol/ALG5* in wild-type embryos (lateral views in B,C, and a dorsal view in D). In cellularizing embryos (B), maternal *ALG5* RNA is ubiquitous. At stage 11, zygotic exression begins in salivary gland precursor cells (arrowhead, C). Strong expression in salivary glands and part of the proventriculus is detected in stage 16 embryos (D). (**E**) Western blot analysis of carboxypeptidase Y (CPY). CPY glycoforms lacking one (–1) or two (–2) oligosaccharide chains can be detected in a *Δalg5* mutant yeast strain. Transformation of yeast (pScALG5) or fly ALG5 (pDmALG5) cDNA restores CPY glycosylation, whereas fly ALG5 cDNAs with either the wol^1 or wol^2 mutations fail to do so.

wol encodes a UDP-glucose:dolichyl-phosphate glucosyltransferase

Using SNP markers, we mapped the *wol* locus to a 48 kb interval on chromosome 2. We sequenced the exons from the nine genes in this interval from *wol* homozygous larvae. In the wol^1 allele we found an A-to-T transversion, and in the wol^2 allele a G-to-A transition in the gene annotated as CG7870 (Fig. 3A). CG7870 is predicted to encode a 326 amino acid protein – the UDP-glucose:dolichyl-phosphate glycosyltransferase ALG5 (Heesen et al., 1994). In wol^1, there is a R209W substitution within the glycosyltransferase domain, whereas W316 is changed to a stop in wol^2. The lethality of *wol* mutants could be rescued by a transgene containing the *ALG5* genomic locus. Whole-mount in situ hybridization showed that *wol* RNA is maternally contributed and expressed zygotically in the salivary glands and proventriculus (Fig. 3B-D), tissues where a lot of protein secretion takes place.

The ALG5 enzyme is involved in N-linked protein glycosylation by transferring glucose from UDP-glucose to dolichyl-phosphate, a lipid residing in the ER membrane (Runge et al., 1984). This glucose is added to the oligosaccharide chain that is assembled in the ER prior to transfer to the nascent polypeptide by oligosaccharyl transferase (Helenius and Aebi, 2004). Substrate recognition by oligosaccharyl transferase is greatly diminished in the absence of terminal glucoses. Mutations that disrupt ALG5 function are therefore expected to cause an accumulation of hypoglycosylated proteins.

To investigate whether the wol^1 and wol^2 mutations affect the function of ALG5, we performed a complementation assay in *Saccharomyces cerevisiae*. As previously shown, mutations in yeast *alg5* lead to hypoglycosylation of secreted proteins (Heesen et al., 1994). This can be assayed by processing of the carboxypeptidase Y (CPY) protein, a vacuolar protein with four N-linked oligosaccharides. In a *Δalg5* yeast strain, CPY glycoforms lacking one or two oligosaccharide chains accumulate (Fig. 3E). Introduction of yeast or *Drosophila* ALG5 cDNA into the *Δalg5* strain restored the glycosylation phenotype of CPY, whereas cDNAs with the wol^1 or wol^2 mutations failed to do so (Fig. 3E). In a yeast growth assay, ALG5 cDNAs with the *wol* mutations only weakly rescued, or failed to rescue, the growth phenotype (see Fig. S4 in the supplementary material). These results suggest that the *wol* mutations either impair the catalytic activity of the ALG5 protein or lead to protein destabilization.

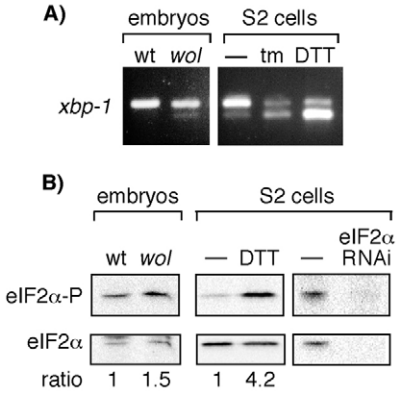

Fig. 4. The unfolded protein response (UPR) is triggered in *wol* embryos. (**A**) Splicing of *xbp1* mRNA was examined by RT-PCR. In wild-type *Drosophila* embryos, only the unspliced 127 bp product was detected, whereas some spliced 104 bp *xbp1* message was found in wol^1 embryos. In untreated S2 cells, mostly unspliced product is present. Tunicamycin (tm) or DTT treatment shifts the ratio of spliced/unspliced in favor of the spliced product. (**B**) Increased eIF2α phosphorylation in *wol* embryos. Western blot analysis of phosphorylated and total levels of eIF2α. The ratio of phosphorylated/total eIF2α was calculated from two independent experiments.

The unfolded protein response is triggered in *wol* mutant embryos

An important function of N-linked glycosylation is to aid the folding of proteins in the ER (Helenius and Aebi, 2004). Accumulation of unfolded proteins in the ER induces the UPR that activates the IRE1 endoribonuclease. In *Drosophila*, this leads to the removal of a 23 bp intron from *xbp1* mRNA in the cytoplasm (Plongthongkum et al., 2007; Ryoo et al., 2007; Souid et al., 2007), which generates a translational frameshift that gives rise to transcriptionally active Xbp1 protein (Plongthongkum et al., 2007).

We examined *xbp1* splicing in *wol* mutant embryos by RT-PCR. Fig. 4A shows that one band with the size expected from unspliced *xbp1* mRNA is obtained from wild-type embryos, whereas both spliced and unspliced products were detected in *wol* embryos. To confirm that this band corresponds to ER-stress-induced *xbp1* splicing, we isolated mRNA from untreated, tunicamycin- or DTT-treated S2 cells. Tunicamycin inhibits N-linked glycosylation, whereas DTT prevents thioester bond formation, and both treatments generate unfolded proteins in the ER. As shown in Fig. 4A, these drug treatments resulted in more of the smaller, spliced *xbp1* mRNA than was found in untreated S2 cells. We conclude that the UPR is triggered by tunicamycin and DTT, as well as by mutations in *wol*.

Another branch of the UPR is activation of the kinase PERK that results in eIF2α phosphorylation and attenuation of translational initiation (Kaufman, 2004). *Drosophila* PERK (also known as PEK) is maternally contributed to the embryo (Pomar et al., 2003). As shown by the western blot in Fig. 4B, a 1.5-fold increase in eIF2α phosphorylation was detected in *wol* embryos as compared with wild type, whereas the total amount of eIF2α remained unchanged. As a control, we prepared protein extracts from S2 cells treated with DTT or with *eIF2α* double-stranded RNA. eIF2α phosphorylation was increased by DTT, whereas both the eIF2α and the phospho-eIF2α bands disappeared in extracts from eIF2α RNAi-treated cells (Fig. 4B).

Taken together, these results confirm that the UPR is activated in *wol* mutants, and indicate that translation might be attenuated in *wol* embryos. We propose that this causes the observed patterning defects. According to this model, reduced maternal *wol* activity would lead to accumulation of unfolded proteins in the ER in early embryos, with a consequent transient increase in eIF2α phosphorylation. Translation of selected maternal mRNAs, including *cad* and the activator of *dpp* expression, would be particularly sensitive to increased eIF2α phosphorylation. Reduced amounts of these transcription factors result in disruption of posterior segmentation and of dorsal-ventral patterning. Although the UPR plays important developmental and physiological roles in *C. elegans*, *Drosophila* and mammals (Ryoo et al., 2007; Shen et al., 2001; Shen et al., 2005; Souid et al., 2007; Wu and Kaufman, 2006), this is the first report to indicate that inappropriate UPR activation may disrupt embryonic patterning.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1745

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