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Developmental Biology 313 (2008) 802–813
DEVELOPMENTAL BIOLOGY
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Establishment of cell fate during early *Drosophila* embryogenesis requires transcriptional Mediator subunit dMED31

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Received for publication 31 May 2007; revised 25 October 2007; accepted 17 November 2007

Abstract

During early *Drosophila* embryogenesis, formation of the anterior–posterior (A/P) axis depends on spatial gradients of maternal morphogens. It is well recognized that positional information is transmitted from these morphogens to the gap genes. However, how this information is being transmitted is largely unknown. The transcriptional Mediator complex is involved in the fine tuning of the signaling between chromatin status, transcription factors and the RNA polymerase II transcription machinery. We found that a mutation in the conserved subunit of the Mediator complex, dMED31, hampers embryogenesis prior to gastrulation and leads to aberrant expression of the gap genes *knirp*s and *Krüppel* and the pair-rule genes *fusi tarazu* and *even-skipped* along the A/P axis. Expression of the maternal morphogens *dorsal* and *hunchback* was not affected in *dMED31* mutants. mRNA expression of *dMED31* exactly peaks between the highest expression levels of the maternal genes and the gap genes. Together, our results point to a role for dMED31 in guiding maternal morphogen directed zygotic gap gene expression and provide the first *in vivo* evidence for a role of the Mediator complex in the establishment of cell fate during the cellular blastoderm stage of *Drosophila melanogaster*.

Keywords: A/P polarity; Mediator complex; Cell fate; dMED31

Introduction

Proper fine tuning of the eukaryotic transcriptome depends on numerous *cis* and *trans* acting factors that modulate the chromatin environment of genes and influence the RNA polymerase II (RNAPII) transcription machinery. The Mediator complex is a core processor in the signaling between RNAPII and transcription factors. This complex is an evolutionary conserved protein assembly of 25–30 subunits (Boube et al., 2002; Bourbon et al., 2004; Guglielmi et al., 2004; Linder and Gustafsson, 2004; Blazek et al., 2005) which was first identified in budding yeast (Flanagan et al., 1991) and consists of four large modules; head, middle, tail and an Srb8–11 module (Asturias et al., 1999; Borggrefe et al., 2002; Samuelsen et al., 2003; Guglielmi et al., 2004).

Support for a specific role during development of Mediator subunits is provided by several studies in *Drosophila melanogaster* (Boube et al., 2000; Gim et al., 2001; Treisman, 2001; Garrett-Engele et al., 2002; Janody et al., 2003; Loncle et al., 2007) and *Caenorhabditis elegans* (Kwon et al., 1999; Zhang and Emmons, 2001; Howard and Sundaram, 2002; Yoda et al., 2005). These studies describe mostly functions of Mediator subunits during late developmental stages, but a role of the subunits during early embryonic development is largely unknown. The Mediator consists of more then 25 subunits, pointing to a multifaceted role of this complex during metazoa development. Understanding this complexity starts with the identification of the function of each subunit.

D. melanogaster MED31 was identified by bioinformatics analysis (Boube et al., 2000) and its presence in the Mediator complex was confirmed in purified complexes from embryos and cells (Park et al., 2001; Gu et al., 2002). In a pull down assay, the Mediator (containing dMED31) complex binds to the transcription factors bicoid (bcd), Krüppel (Kr), fushi tarazu (ftz), dorsal (dl) and HSF, but not twist (twi), hunchback (hb) and even-skipped (eve) (Park et al., 2001). Moreover, the Mediator complex is required for *in vitro* transcription from

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doi:10.1016/j.ydbio.2007.11.019

developmentally important promoters regulated by these transcription factors. Despite these *in silico* and *in vitro* results, to date the functional role of MED31 in eumetazoa remains elusive. Here we report the identification of the highly conserved *Drosophila* Mediator subunit dMED31 as a novel maternal-effect gene necessary for proper segment specification during early embryogenesis. *dMED31* mutant females have fecundity defects and embryos deposited by homozygous mothers display severe defects along the anterior–posterior (A/P) axis when gastrulation is initiated. Whereas expression of maternal morphogens is not affected, alterations in gap and pair-rule gene expression during the proceeding blastoderm stage correlate with these defects observed in *dMED31* mutant embryos. Remarkably, a small percentage of the progeny of homozygous mutant females escape from embryonic death and develop into adults. These escapers have defects in their abdominal segmentation pattern, a phenotype enhanced by mutations in *dMED13*. Our findings provide the first *in vivo* evidence for a specific role of *dMED31* in establishing cell fate in the cellular blastoderm and point to a role for the Mediator in guiding maternal morphogen directed zygotic gap gene expression.

Material and methods

Drosophila stocks and genetics

Fly stocks were maintained at 22 °C according to standard protocols. The y^1w^{1118} line was used for wild-type (wt) preparations. The E709 stock was a generous gift of A. Ephrussi (Heidelberg, Germany). The $dMED17^1$ and $dMED13^1$ P element insertion lines are previously described (Boube et al., 2000) and were obtained from the Bloomington stock center (Indiana University, USA). P element excision lines were generated with the Δ2–3 transposase (Robertson et al., 1988). From a collection of approximately 75 individual excision lines, a back to wt allele ($dMED31^{21}$) was isolated. Genomic DNA surrounding the P element insertion site was sequenced to confirm precise excision.

dMED31 mapping and transgene construction

Plasmid rescue analysis was performed to recover the 3′ and 5′ flanking genomic sequences of the pLacW insertion site (Guo et al., 1996). Subsequent DNA sequencing and database searches revealed the exact genomic position of the P element. PCR analysis combined with complementation tests by means of standard crosses to deficient chromosomes encompassing the identified cytological position was used to confirm the localization of the P element and to confirm a link between the P element insertion and the female fertility phenotype of the E709 mutant. A genomic fragment containing the dMED31 locus (CG1057), its preceding intergenic region together with approximately 250 bp of genomic DNA flanking the polyadenylation site was PCR amplified with primer pair 5′-GAAGGATCCGCGCATCACATTAGGGGTAG-3′ and 5′-AGCGAATTCTGTGGGTCTGTGAATTCAC-3′, cut with *Bam*HI and *Eco*RI and cloned in an identically digested pCasper4-Pme1 provided by L.G. Fradkin (Leiden, The Netherlands). A transgenic fly carrying the *P[dMED31]* transgene on the second chromosome was created by Genetic Services Inc. (Sudbury, USA) and was crossed into the E709 strain. Single fly PCR analysis was used to confirm its presence (Gloor et al., 1993).

Assessment of embryonic viability and abdominal defects

To assay embryonic viability, embryos were collected (0–6 h) on apple juice plates and counted, and the hatch rate was determined by visual inspection of the egg cases 2 days after egg laying. Female fecundity was assayed by crossing 10 1-week-old females with 5 males in standard food vials containing yeast paste. After 3 days, flies were transferred to vials without yeast granules/paste and the amount of embryos deposited after 20 h was determined. Groups were compared by Student's *t*-test. Inspection of abdominal defects was carried out by visual inspection of 2–5 day-old flies. Images were captured with a Olympus BX50 light microscope.

Immunofluorescence

For these studies, embryos deposited by $dMED31^{1/1}$ mothers crossed with wt males or embryos deposited by wt females crossed with wt males were used. Isolation, fixation and immunolabeling of embryos were performed as described (Theurkauf, 1994). Blocking of embryos and labelings were performed in PBS +0.3% TritonX-100+5% BSA. Primary antibodies included mouse anti-pH3S10 (1:200, Cell Signaling), rabbit anti-tailless (1:200, #812) obtained via the Asian Distribution Centre for Segmentation Antibodies (Kosman et al., 1998), rabbit anti-hunchback (1:1000), rabbit anti-Krüppel (1:500) and rabbit anti-knirps (1:50) (generously provided by H. Jäckle and P. Carrera, Göttingen, Germany) and concentrated supernatants of mouse anti-even-skipped (1:5) (3C10 developed by C. Goodman) and mouse anti-dorsal (1:5) (7A4 developed by R. Steward) obtained from the Developmental Studies Hybridoma Bank (Iowa, USA). Secondary antibodies included FITC-conjugated goat anti-mouse or goat anti-rabbit IgGs (Jackson ImmunoResearch). Embryos were incubated in a 0.2 μg/ml DAPI solution in PBS to visualize the DNA, mounted in citifluor (Agar scientific) and analyzed by FM, LM (Carl Zeiss Axioskop2) or CLSM (Leica TCS SP2). Captured images were processed using Paint Shop Pro.

In situ hybridization

For *in situ* hybridization experiments, embryos deposited by $dMED31^{1/1}$ mothers crossed with either $dMED31^{1/1}$ or $dMED31^{1/+}$ males were pooled and compared to embryos deposited by wt mothers crossed with wt males. Hybridizations were essentially carried out as described (Tautz and Pfeifle, 1989). Plasmids for probe production against *Krl* (pT:Kr) (Knipple et al., 1985), *kni* (pcJ15) (Pankratz et al., 1990), *eve* (p48-X1.4) (Macdonald et al., 1986), *ftz* (pT-ftz) (Ish-Horowicz and Pinchin, 1987) and *en* (pen) were generously provided by P. Gergen (Stony Brook, USA). Digoxigenin (DIG) incorporated RNA probes were generated from linearized plasmids by *in vitro* transcription in the presence of a DIG RNA labeling mixture (Roche). Hybridizations were carried out at 55–60 °C and the probes were detected with anti-digoxigenin-AP Fab fragments (Roche). Chi square tests were used to compare the percentages of abnormal mRNA expression in control versus *dMED31* mutant stage 3–8 embryos. The quantified data represent the total amount from 3 independent hybridization experiments.

Results

Mutations in dMED31 cause defects in fecundity and embryogenesis

We analyzed libraries of existing single P element insertions in *D. melanogaster* for novel genes involved in embryogenesis. From a collection of mutants (kindly provided by A. Ephrussi) that affect female fertility, we recovered a single P element insertion line E709 ($dMED31^1$, see below) as a candidate for further investigation. Embryos deposited by homozygous females crossed with wild-type (wt) males (further referred to as mutant embryos) rarely hatched (2.1%) compared to embryos deposited by wt females (88.2%) further referred to as wt embryos (Table 1). Unless otherwise specified, all studies in this manuscript were performed with embryos derived from females that were crossed with wt males. In the mutant, the P element was mapped within the 5′-UTR of CG1057 (Fig. 1A) and its

Table 1
Mutations in *dMED31* disrupt female fecundity and embryonic viability

Genotype female	Hatching rate (%)	Fecundity (eggs/20 h±SEM)
y^1w^{1118}	88.2 (n=1005)	46.0±3.3 (n=45 cohorts)
$dMED31^{1/1}$	2.1 (n=574)	7.4±1.5 (n=42 cohorts)
$dMED31^1/Df(3R)Z1,ry^*$	3.2 (n=632)	3.2±0.4 (n=51 cohorts)
$dMED31^{1/21}$	91.6 (n=1287)	ND
$P[dMED31];dMED31^{1/1}$	57.9 (n=401)	ND

Flies of indicated genotype were crossed with wt males. Hatching of n embryos was monitored and the fecundity of n cohorts of 10 females was determined.

location was confirmed by PCR mapping (not shown). This gene encodes the fly homologue of the *S. cerevisiae* suppressor of HPR1 (SOH1) and is part of the transcriptional Mediator complex (Boube et al., 2000; Park et al., 2001). SOH1 is also known as TRAP18, but recently unified nomenclature designated the protein MED31 (Bourbon et al., 2004), therefore the mutant will be referred to as $dMED31^1$. The *dMED31* locus encodes two alternative transcripts as evident from the expressed-sequence-tagged database (www.fruitfly.org), which both encode the full length *Drosophila* dMED31 protein (Fig. 1C). PCR analysis and rapid amplification of cDNA ends (RACE) analysis of cDNA isolated from $dMED31^{1/1}$ females revealed the presence of P-lacW specific sequences in the 5′-UTR of the *dMED31* transcript, indicating that transcription originates from the P element (not shown). As the transposon landed in the first intron of CG1057-RA, exactly after the first splice donor site of CG1057-RB, it is likely that appropriate splicing and production of CG1057-RB do not occur (Fig. 1B). Therefore, we conclude that the P element insertion in the *dMED31* mutant alters dMED31 mRNA structure and production.

In addition to poor hatching rates of embryos deposited by $dMED31^{1/1}$ females, mutant females also displayed fecundity defects and cohorts of 10 $dMED31^{1/1}$ females produced only 7.4±1.5 eggs/20 h ($p<0.0001$) compared to 46.0±3.3 eggs deposited by wt females (Table 1). In order to confirm linkage between the observed mutant phenotype and the P element insertion, $dMED31^1$ was placed over the deficiency *Df(3R)Z1, ry**, in which at least 82 genes surrounding the *dMED31* locus are deleted. Transheterozygous $dMED31^1/Df(3R)Z1,ry^*$ females showed more severe fecundity defects ($p<0.05$)

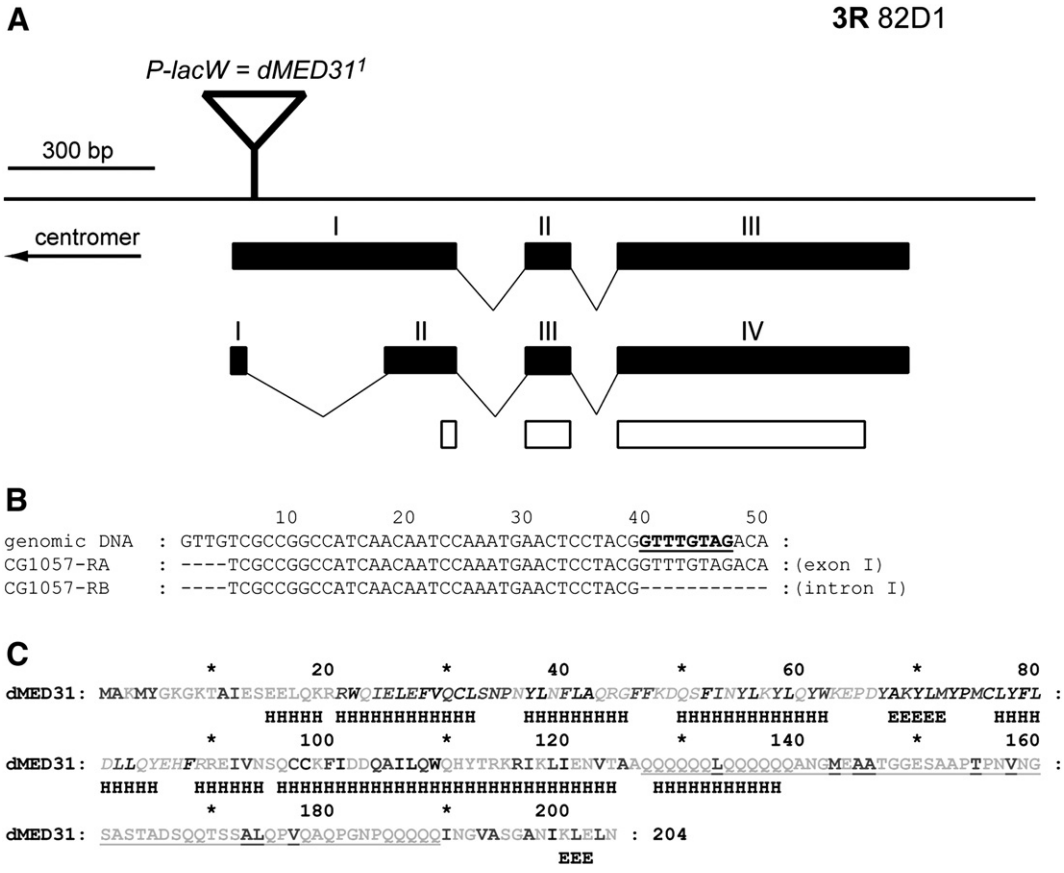


Fig. 1. Mutations in *dMED31* disrupt early embryogenesis. (A) The region depicted comprises cytological position 82D1 of chromosome 3R and harbors CG1057, which encodes 2 mRNAs that both encrypt a 204 amino acid ORF that represent the fly MED31 protein. The position of the P-lacW insertion allele $dMED31^1$ is indicated (triangle). Transcripts are indicated by black boxes and their encoded proteins are depicted underneath (white boxes). (B) The transposon landed within the 5′-UTR of the CG1057-RA messenger exactly after the first splice donor site of intron I of the alternative CG1057-RB mRNA (the 8 bp insertion site is underscored/bold letter type). (C) The nuclear 204 amino acid dMED31 protein displays a strong helical character (predicted extended (E) and helical (H) secondary confirmations are denoted under the primary sequence). Gray values represent the calculated solvent accessibility (black represents 0% exposure). Residues 21 to 89 (italics) represent a core region of 69 amino acids that is highly conserved (Linder and Gustafsson, 2004). The COOH terminal half of dMED31 harbors a solvent accessible glutamine (Q) rich stretch (underscored). Calculations were performed with software available from the ExPASy Molecular Biology Server.

compared to *dMED31*$^{1/1}$ females (Table 1). A small percentage of embryos derived from *dMED31*$^{1/1}$ mothers is able to hatch and a small percentage of these larvae is able to reach the adult stage (see also below). A small percentage (3.2%) of embryos derived from transheterozygous *dMED31*^{1}*/Df(3R)Z1,ry** mothers was also able to hatch; however, none of these larvae reached the adult stage. Based on these observations, we conclude that the *dMED31*^{1} allele is hypomorphic. Next, we restored the *dMED31*^{1} insertion allele by remobilizing the P element (Robertson et al., 1988). When we placed *dMED31*^{1} over a precise excision allele *dMED31*^{21}, the hatching rate of embryos deposited by *dMED31*$^{1/21}$ mothers was restored to 91.6% (Table 1). Finally, when we introduced a genomic fragment that harbors the entire *dMED31* locus including its flanking intergenic regions (*P[dMED31]*), the hatching rate was restored to 57.9% (Table 1). Together these results show that an intact *dMED31* allele is required for female fecundity and intact function of maternal dMED31 is required for embryonic viability.

Establishment of embryonic A/P polarity requires Mediator subunit dMED31

In order to determine at which stage embryogenesis is disrupted in eggs deposited by *dMED31*$^{1/1}$ females, embryos derived from wt and *dMED31*$^{1/1}$ females were labeled with an antibody directed against serine 10 phosphorylation of histone H3 (pH3Ser10), which enabled us to visualize mitotic domains within the developing embryo. Close inspection of these mitotic domains in combination with morphological analysis revealed that mutant embryos reach the cellular blastoderm stage (Figs. 2F, G), but begin to display severe defects upon gastrulation (Figs. 2H, I). During wt gastrulation and germ band elongation, cells associated with the migrating polar plate and stomodeum are highly proliferating (Figs. 2C, D). In mutant embryos, these mitotic domains were missing or completely mislocalized and coincided with altered embryonic morphology. The formation of the posterior located pole cells appeared unaffected in mutants (Fig. 2L) but, quickly after their formation cells at the anterior and posterior pole started to delaminate toward the interior of the embryo (Fig. 2N). Normally, pole cells migrate during gastrulation; first over the dorsal surface, then the pole cells migrate inwards the embryo to the posterior end, where they form the future reproductive system. Upon gastrulation, the formation of the major invaginations (proctodeal invagination, transverse furrow, stomodeal invagination and the cephalic furrow) was observed in *dMED31* mutant embryos (Fig. 2G). Nevertheless, cells associated with the anterior and posterior pole of mutant embryos frequently displayed abnormal migratory behavior. During subsequent embryonic stages, abnormal cell migration and degeneration at both poles eventually resulted in severe polarity problems within the developing embryo. Especially the cells that accompany the migrating polar plate behaved abnormal (Fig. 2). Moreover, cell loss was frequently more profound at the dorsal-posterior region of mutant embryos. Similar defects were found in embryos deposited by *dMED31*^{1}*/Df(3R)Z1,ry** or *dMED31*$^{1/1}$ females crossed with *dMED31*$^{1/1}$ males. The majority of the embryos deposited by *dMED31*^{1}*/Df(3R)Z1,ry** or *dMED31*$^{1/1}$ mothers (independent of the paternal genotype) displayed severe morphological defects beyond st.8 (estimated >90%). Thus, a mutation in the *dMED31* locus causes a maternal effect phenotype in over 90% of the embryos and leads to aberrant cell migration and impaired anterior–posterior (A/P) axis formation in the embryo, suggesting that dMED31 is an essential factor required for establishing cell fate in the cellular blastoderm.

Mediator subunit dMED31 is necessary for zygotic gap and pair-rule gene transcription

Previously, *in vitro* experiments demonstrated that the *Drosophila* Mediator complex is able to bind developmental transcription factors and is essential for the *in vitro* transcription from promoters regulated by these transcription factors (Park et al., 2001). Moreover, immunoprecipitation of embryonic nuclear extracts with an antibody directed against dMED31 abolished Mediator composition and affected *in vitro* RNA transcription, suggesting that the Mediator complex is required to control gene transcription during early development (Park et al., 2001). To test this suggestion, we first analyzed expression profiles of all Mediator genes using available data sets (www.fruitfly.org; (Tomancak et al., 2002). These data show that the entire Mediator complex is maternally supplied to the embryo and is highly expressed prior to gastrulation (see Supplementary Fig. 1), indicating that this complex is required at this specific stage during early embryonic development. Comparing the expression profile of the Mediator with expression profiles of maternal genes and gap genes revealed that expression levels of Mediator genes exactly peak between highest expression levels of maternal genes and gap genes during development (see Supplementary Fig. 1), suggesting a specific function of the Mediator complex at this specific moment.

Upon egg deposition, maternally contributed morphogen gradients of genes such as; *dl*, *cad*, *nos*, *bcd* or *hb* define the embryo's polarity, the dorsal–ventral (D/V) axis and the A/P axis (Rivera-Pomar and Jackle, 1996). Because expression of dMED31 peaks at a time point later than the maternally supplied morphogens, we hypothesized that localization and expression levels of these morphogens in dMED31 mutants are not affected. In agreement with this hypothesis, the localization and the expression of the maternal morphogens dl and hb were unaffected in embryos deposited by *dMED31*$^{1/1}$ mothers (Fig. 3E).

During wt embryogenesis, *cad*, *nos*, *bcd* and *hb* regulate expression of the gap genes *tailless* (*tll*), *giant* (*gt*), *knirps* (*kni*) and *Kr* (Hulskamp et al., 1990; Kraut and Levine, 1991). dMED31 expression peaks before high expression levels of the gap genes (see Supplementary Fig. 1) which coincided with the timing of the morphological abnormalities observed in *dMED31* mutant embryos (Fig. 2). These data suggest that gap gene expression may be affected in *dMED31* mutant embryos. To investigate whether *dMED31*^{1} indeed affects embryonic gap gene expression, we analyzed mRNA and

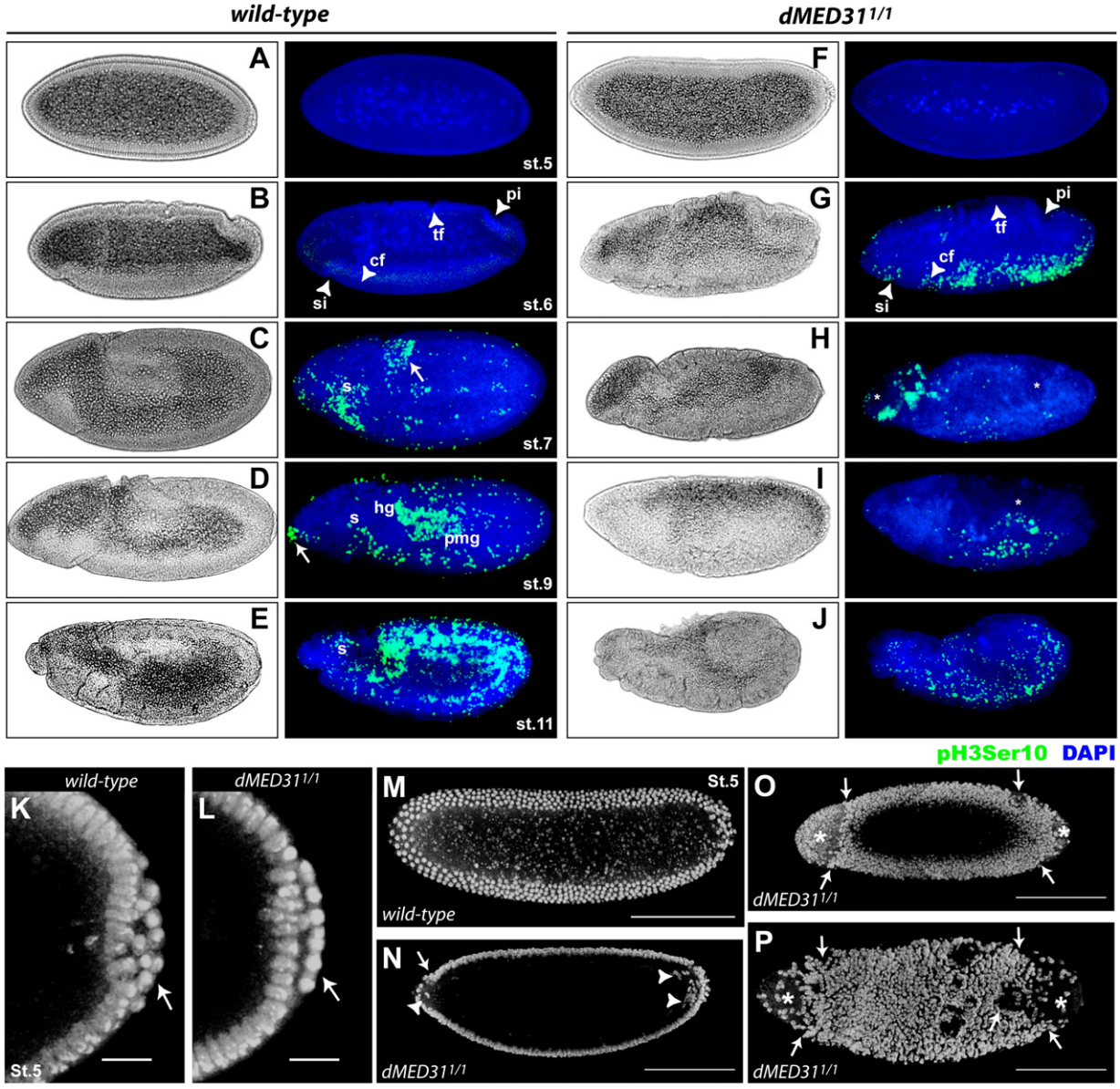


Fig. 2. Mitotic domains associated with migrating cells during gastrulation are mislocalized or absent in *dMED31* mutant embryos. Embryos deposited by wt (A–E) and *dMED31*$^{1/1}$ (F–J) females crossed with wt males were analyzed by LM and FM. Embryos were labeled with an antibody against pH3S10 (green) and counterstained with DAPI (blue) to visualize mitotic cells and the DNA, respectively. Images represent lateral views with the anterior to the left. Stages of embryonic development are indicated. (A) Wt embryo at the syncytial blastoderm stage. Yolk nuclei are visible within the embryo. (B) Upon early gastrulation morphologically several invaginations can be identified. (pi) Proctodeal invagination; (tf) transverse furrow; (si) stomodeal invagination; (cf) cephalic furrow. (C) During germ-band elongation, cells associated with the migrating polar plate (arrow) and stomodeum (s) are highly proliferating. (D) At the extended germ-band elongation stage, cells at the anterior midgut primordium (arrow), hindgut primordium (hg) and posterior midgut rudiment (pmg) are proliferating and the parasegments become visible. (E) The parasegmental furrows are being formed on the dorsal region of the developing embryo and subdivide the retracting germ-band into metameric units. (F) *dMED31* mutant embryos reach the syncytial blastoderm stage and (G) the formation of the major invaginations during gastrulation can be observed. (H, I) After gastrulation has initiated, mitotic domains become mislocalized and embryos develop abnormal regions that lack cells (asterisks). (J) Progression into late stage embryogenesis eventually results in morphologically abnormal embryos. Note that especially cells associated with the migrating polar plate behave abnormal and result in severe deformations at the posterior-dorsal region of the embryo. (K–P) Wt and *dMED31* mutant embryos deposited by homozygous females were labeled with DAPI to visualize the DNA and analyzed by CLSM. Anterior is to the left. (K and L) During st.4–5 of embryonic development, the pole cells are formed at the posterior pole of the embryo. (L) In *dMED31* mutant embryos, pole cell formation is normal, (N) but during gastrulation, cells associated with the anterior and posterior pole start to delaminate toward the interior of the embryo (arrowheads) and abnormal cell ingression can be observed at the anterior-dorsal region of the embryo (arrow). (O, P) During later stages, abnormal migration (arrows) and degeneration at the poles result in cell loss (asterisks). Scale bars: 20 μm (K, L), 150 μm (M–P).

protein distribution of the gap genes *kni* and *Kr* (Fig. 3A). Early mRNA expression of these genes can be observed at the anterior tip and as a band in the center of wt embryos. Furthermore, *Kr* expression is also strong at the posterior end of wt embryos during gastrulation and germband elongation. These patterns of mRNA expression change during subsequent embryonic stages. The protein expression of both genes globally parallels their mRNA expression pattern. In mutant embryos deposited by

dMED31$^{1/1}$ females, *kni* and *Kr* mRNA expression during st.4–5 is much lower compared to wt embryos (Fig. 3A). Moreover, abnormal mRNA and protein expression patterns of these two genes were observed during early stages of embryogenesis. Interestingly, the polar expression of *kni* and *Kr* is hardly detectable or even absent. *dMED31*$^{1/1}$ embryos also displayed abnormal patches of *kni* expression in cells that reside within the ventral region (Fig. 3A), suggesting that these cells received incorrect information from the maternal morphogens. The central band of the Kr protein is sometimes smaller in mutant embryos compared to wt Kr expression and high Kr expression could also be found in cells outside the central band. Overall 44.6% of *dMED31*$^{1/1}$ embryos deposited by homozygous mutant females displayed abnormal *Kr* mRNA expression and 18.1% of the embryos displayed abnormal *kni* expression (Fig. 3D).

The maternal terminal system controls the restricted expression of the terminal gap genes *tll* and *huckebein* (*hkb*) in the embryonic termini via the localized activation of the Torso (Tor) receptor tyrosine kinase signaling pathway (Klingler et al., 1988; Weigel et al., 1990; Bronner and Jackle, 1991). Because dMED31 mutant embryos show terminal defects, we analyzed protein expression of *tll*, whose activity is required to specify cell fates at the embryonic poles (Strecker et al., 1986). Protein expression of tll was unaffected in embryos deposited by *dMED31*$^{1/1}$ females compared to wt. However, as previously noted, we frequently found gaps in the cellular blastoderm at both termini and we quantified the amount of st.5 embryos in which posterior located tll expressing cells were missing (Fig. 3C). In 28.0% (n=82) of st.5 embryos produced by *dMED31*$^{1/1}$ mothers, posterior located tll expressing cells were missing (arrows), which was 3.1% in wt embryos (n=96). These data suggest that dMED31 is not required for the activation of the terminal gap genes in response to Tor signaling at the embryonic termini, but dMED31 is required to maintain cell viability at the termini.

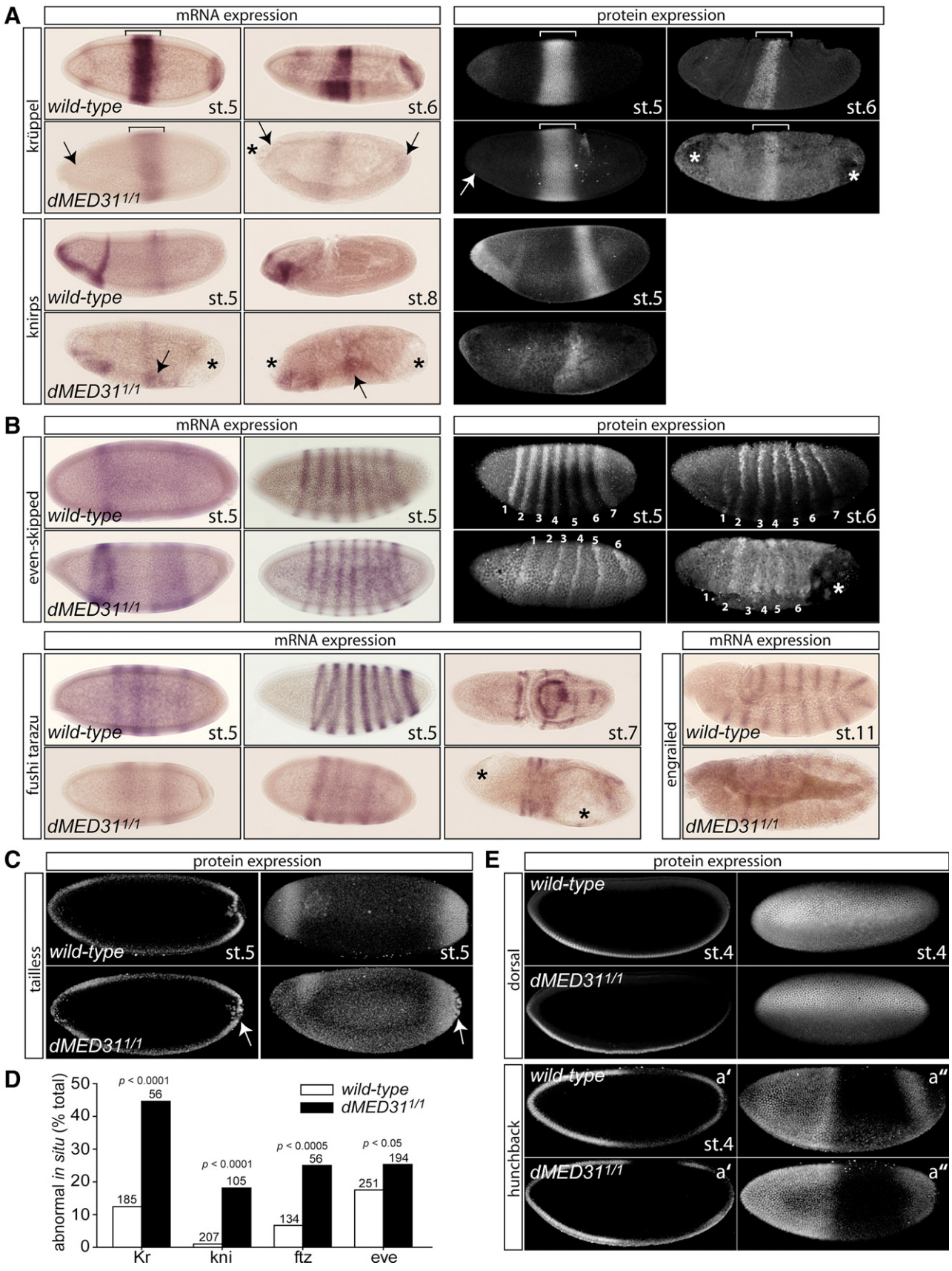
In wt embryos, gap gene expression proceeds pair-rule gene expression. Pair-rule gene patterning occurs in 7 distinct stripes. *Eve*, *ftz* and other pair-rule genes provide cell identity to cells in the blastoderm stage. In 25% of the embryos deposited by *dMED31*$^{1/1}$ mothers, the mRNA expression of *ftz* is lower and abnormal compared to wt embryos (Figs. 3B, D). It should be noted that abnormalities in *ftz* expression are mainly observed during stages 6–8 and *ftz* expression seems not severely altered in earlier stages. Similar results were obtained when expression patterns of *eve* were analyzed in mutant embryos and compared to wt embryos. Wt mRNA expression of *eve* is initially blurred, but rapidly resolve into sharply defined stripes. In the cellular blastoderm of mutant embryos, only minor alterations in mRNA expression of *eve* could be observed (Figs. 3B, D) during early stages. The availability of an antibody against Eve allows the analysis of protein expression patterns and this analysis sometimes revealed less than the normal 7 stripes (Fig. 3B). Comparable to abnormalities in *ftz* expression, abnormal expression patterns of *eve* mRNA were most obvious in later stages (stages 6–8) (Fig. 3C, data not shown). Based on these observations, we conclude that in *dMED31*$^{1/1}$ mutant embryos, expression of *eve* and *ftz* is affected. Although at this point, we cannot distinguish whether the observed abnormalities are due to primary defects in dMED31 mediated *eve* and *ftz* expression or due to embryonic morphological alterations induced by a mutation in dMED31.

Expression of the segment polarity and Hox genes is activated by the pair-rule genes and a subset of the gap genes during late stage embryogenesis (>st.10). We also analyzed the expression of the segment polarity gene *en* whose expression is restricted to 14 stripes along the A/P axis. Only a limited number of mutant embryos could be scored positive for *en* expression and did not allow quantification (Fig. 3B). Likely late stage *en* expression could not be detected in *dMED31*$^{1/1}$ mutant embryos due to severe defects during gastrulation. Thus, although the morphological abnormalities (Fig. 2) are uniform and observed in over 90% of mutant embryos, it is more complicated to define precisely the abnormalities in zygotic gene expression because of variability (Fig. 3). Despite this, our results demonstrate that the dMED31 subunit is required to establish domains of gap and pair-rule gene expression along the A/P axis during embryogenesis.

Mutations in Mediator subunits dMED31 and dMED13 abrogate abdominal segmentation

During our analysis of the embryonic hatching rate of eggs deposited by *dMED31*$^{1/1}$ females, we found that 2.1% (n=574) of the embryos was able to hatch. Moreover, we noticed that some of these larvae could also progress through subsequent developmental stages and produced viable adults. Interestingly, morphological analysis of the adult survivors revealed the presence of deformations in abdominal segmentation (Figs. 4A–G, Table 2) and we used these abdominal defects as a quantifiable marker for further analysis of dMED31 function during development. When *dMED31*$^{1/1}$ females were crossed with wt males 33.7% of the cohort of rare escapers that reached the adult stage developed segmentation defects, while this percentage was 45.6% when *dMED31*$^{1/1}$ females were crossed with *dMED31*$^{1/1}$ males. Mutant flies of both sexes displayed features of incomplete tergite separation, showed the formation of additional tergites, developed distinctive patches of pigment in the abdominal epidermis, had abnormally positioned sternites or lacked halteres (Figs. 4B, C, E, G). These defects were not present in flies that carried either one or two copies of the *P [dMED31]* transgene. Transheterozygous *dMED31*^{1}*/Df(3R)Z1, ry** or *dMED31*$^{1/1}$ adults derived from heterozygous parents did not develop significant abdominal deformations (Table 2). Together, this indicates that abnormal maternally supplied *dMED31* results in segmentation defects and this effect is enhanced due to aberrant zygotic expression of *dMED31*. Furthermore, since adults produced by *dMED31*^{1}/+ mothers did not develop segmentation defects (Table 2), the *dMED31*^{1} allele is recessive.

We wondered whether mutations in other Mediator subunits also caused segmentation defects during early embryogenesis. Previously it was shown that the dMED13 and dMED17 Mediator subunits are required for segment identity


A
mRNA expression
protein expression
krüppel
knirps
wild-type
dMED31 1/1
st.5
st.6
st.8
B
even-skipped
fushi tarazu
engrailed
st.7
st.11
C
protein expression
tailless
D
abnormal in situ (% total)
wild-type
dMED31 1/1
p < 0.0001
p < 0.0005
p < 0.05
185
56
207
105
134
56
251
194
Kr
kni
ftz
eve
E
dorsal
hunchback
a′
a″

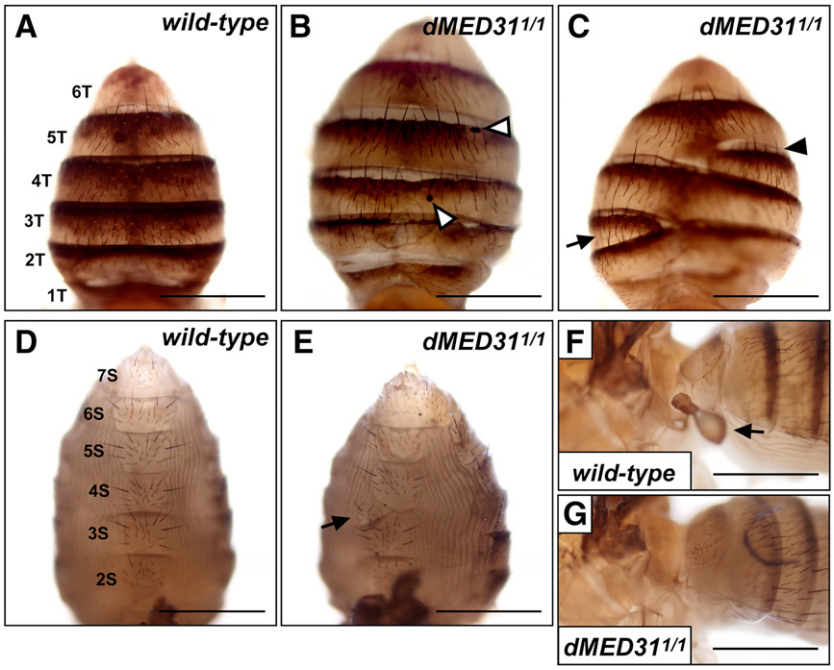


Fig. 4. *dMED31* mutants develop abdominal deformations. Abdominal segmentation was investigated in wt and $dMED31^{1/1}$ adult flies derived from homozygous mothers. (A–C) Dorsal, (D–E) ventral and (F–G) lateral views of the abdomen from wt (A, D, F) and $dMED31^{1/1}$ (B, C, E, G) females. (A) Wt flies have a distinctive pattern of tergites (T1–6). $dMED31^{1/1}$ flies derived from homozygous parents develop: (B) patches of pigment (boxed arrowheads) (14.3%, $n=31$), (C) incomplete tergites (black arrowheads) and additional tergites (arrow) (13.4%, $n=29$). (D) The ventral abdomen of wt females displays a distinctive pattern of sternites (S2–7). (E) $dMED31^{1/1}$ female in which 4S formation is abrogated (arrow) (4.1%, $n=9$). (F) Lateral view of a wt haltere (arrowhead). (G) Haltere development is frequently disrupted in *dMED31* mutants derived from $dMED31^{1/1}$ mothers (10.6%, $n=23$). All the above-described defects were also found in male mutants. Percentage represents percentage of total and *n* indicates the amount of flies with pigment, tergite, sternite or haltere defects. Scale bars: 500 μm.

specification controlled by the Hox genes *proboscipedia* and *Sex combs reduced* during larval development (Boube et al., 2000). Mutant adults develop abnormal labial palps, while *dMED13* mutants alone also formed ectopic sex comb teeth on the second tarsal segment of the prothoracic (T1) leg (Boube et al., 2000). Since both mutations are lethal, we investigated abdominal segmentation in heterozygous mutants. Heterozygous *dMED17* mutants displayed normal segmentation (not shown); however, when $dMED13^{1}$/+ females were crossed with wt males, 22.7% of the $dMED13^{1}$/+ adult progeny had segmentation defects, while 9.8% of the +/+ offspring had defects (Table 2). This indicates that $dMED13^{1}$ is a dominant modifier of segmentation in *Drosophila*. Most likely abnormal maternal deposition of *dMED13* causes segmentation defects in

Fig. 3. Early expression of cell fate determinants along the A/P axis is disrupted in embryos deposited by $dMED31^{1/1}$ females. Wt embryos (deposited by wt females crossed with wt males) and mutant embryos (deposited by $dMED31^{1/1}$ females crossed with pooled $dMED31^{1/1}$ and $dMED31^{1/+}$ males) were analyzed for expression of cell fate determinants. (A) *In situ* mRNA and protein expression of the gap genes *kni* and *Kr* is abrogated in *dMED31* mutant embryos. At st.5, the Kr mRNA and protein are expressed as a central band in wt embryos. Low expression can be found at both embryonic poles. The polar expression of Kr mRNA becomes stronger during subsequent stages of embryonic development. In embryos deposited by $dMED31^{1/1}$ females, expression of Kr mRNA in the central band can be detected, but is frequently more faint. Late stage polar expression of the mRNAs and proteins is hardly detectable (arrows). Note that some cells outside the central band express elevated levels of the Kr protein and the central band of protein expression sometimes appears smaller compared to the wt (brackets). In wt embryos at st.5, kni mRNA and protein can be detected at the anterior and as a small central band in the embryo. Kni expression becomes restricted at the anterior during gastrulation and germband elongation. *dMED31* mutant embryos express patches of kni at their ventral region (arrows). Moreover, mRNA and protein expression at the anterior of the embryos is reduced, while the central band is frequently faint and twisted. (B) Pair-rule patterning of *eve* and *ftz* in embryos deposited by $dMED31^{1/1}$ and wt mothers. In wt embryos, mRNA expression of eve and ftz is initially blurred, but rapidly resolve into 7 stripes. Early ftz and eve expression in mutant embryos parallels the wt pattern. However, ftz expression is consistently lower compared to wt embryos and becomes abnormal during gastrulation (st.7). Eve mRNA expression is only marginally different in embryos deposited by $dMED31^{1/1}$ compared to wt expression (see panel E), but immunolabelings sometimes revealed the presence of 6 instead of the 7 stripes in *dMED31* mutant embryos. Expression of the segment polarity gene *en* was also investigated. The pattern of 14 stripes along the A/P axis is present in mutant embryos, but is less clear. However, only limited embryos could be scored positive and did not allow quantification. (C) In st.5 wt embryos, the tll protein is expressed at the posterior cap and as a horseshoe-shaped stripe in the anterior domain (Pignoni et al., 1992). The expression of tll is unaffected in *dMED31* mutant embryos. However, we noticed that in st.5 embryos, cells at both termini were frequently missing or delaminating toward the interior of the embryo (see Fig. 2) and in 28.0% ($n=82$) of the embryos deposited by $dMED31^{1/1}$ mothers, posterior located tll expressing cells were missing (arrows), which was 3.1% in wt embryos ($n=96$). (D) Quantification of aberrant Kr, kni, ftz and eve *in situ* mRNA expression. Faint and/or abnormal patterning in st.3–8 embryos were the criteria used to score *n* embryos (depicted at the top of each histogram) for abnormal expression (*p* values as determined by χ^2 analysis). (E) Protein analysis of the maternal morphogens dl and hb revealed no differences in expression and localization between embryos deposited by $dMED31^{1/1}$ females and embryos deposited by wt females; dl is expressed in the ventral blastoderm of st.4 embryos, while hb expression is normally expressed in the anterior half of the embryo and at the posterior (panel a′ represents a single scan of panel a″). In the images, anterior is to the left and asterisks mark areas of cell loss.

Table 2
Mutations in Mediator subunits *dMED13* and *dMED31* disrupt abdominal segmentation

Crossings (genotype female × male)	Adult offspring	% abnormal	*n*
$y^1w^{1118} \times y^1w^{1118}$	+/+	2.5	315
$dMED31^1/+ \times y^1w^{1118}$	$dMED31^1/+$	3.0	291
	+/+	1.9	265
$dMED31^1/TM3 \times dMED31^1/TM3$	$dMED31^{1/1}$	4.6	196
$dMED31^1/TM3 \times Df(3R)Z1,ry*/TM3$	$dMED31^1/Df(3R)Z1,ry*$	1.4	147
$dMED31^{1/1} \times y^1w^{1118}$	$dMED31^1/+$	33.7	196
$dMED31^{1/1} \times dMED31^{1/1}$	$dMED31^{1/1}$	45.6	217
$dMED31^1/Df(3R)Z1,ry* \times y^1w^{1118}$	Larval lethal		
$dMED31^{21/21} \times dMED31^{21/21}$	$dMED31^{21/21}$	0.0	139
$P[dMED31]/+;dMED31^{1/1} \times y^1w^{1118}$	$P[dMED31]/+;dMED31^1/+$ and $dMED31^1/+$	0.0	119
$P[dMED31];dMED31^{1/1} \times P[dMED31];dMED31^1/TM3$	$P[dMED31];dMED31^{1/1}$	8.7	69
	$P[dMED31];dMED31^1/TM3$	6.9	102
$dMED13^1/+ \times y^1w^{1118}$	$dMED13^1/+$	22.7	203
	+/+	9.8	193
$dMED31^{1/1} \times dMED13^1/TM3$	$dMED31^1/dMED13^1$	54.7	53
$dMED13^1/dMED31^1 \times y^1w^{1118}$	$dMED13^1/+$ and $dMED31^1/+$	15.1	205

The total percentage of abdominal defects of *n* flies in the progeny derived from flies of indicated genotype is shown.

the +/+ offspring and these defects are enhanced by aberrant zygotic expression of *dMED13* in the $dMED13^1/+$ progeny.

Next we analyzed whether the $dMED31^1$ allele could enhance the dominant $dMED13^1$ phenotype. When $dMED31^{1/1}$ females were crossed with $dMED13^1/TM3$ males, 54.7% of the $dMED31^{1/}/dMED13^1$ adults had abdominal defects (Table 2), demonstrating that zygotic expression of the $dMED13^1$ allele enhanced the $dMED31^1$ phenotype (χ^2 $p<0.006$ compared to the progeny from $dMED31^{1/1}$ females crossed to wt males). However, no increase in segmentation defects was observed in the progeny from $dMED13^1/dMED31^1$ mothers compared to defects observed in progeny from $dMED13^1/+$ mothers (Table 2), demonstrating that $dMED31^1$ is not an enhancer of $dMED13^1$ when maternally supplied. This indicates that the dMED31 and dMED13 subunits of the Mediator are both required for proper cell fate specification along the embryonic A/P axis.

Discussion

Cell fate specification requires maternally supplied components of the transcriptional Mediator complex

Our findings identify a component of the conserved eukaryotic transcriptional Mediator complex, dMED31, that is required for normal initiation of zygotic gene expression during the blastoderm stage of *Drosophila* embryogenesis. Female flies that carry a mutation in the *dMED31* gene suffer from fecundity defects and the embryos deposited by these females display abnormal embryogenesis due to aberrant cell migration events upon gastrulation. Impaired embryogenesis coincided with changes in *kni*, *Kr*, *ftz* and *eve* expression along the A/P axis. Furthermore, adult flies derived from embryos that escaped from embryonic death displayed severe defects in their abdominal segmentation. Because mRNA production was hampered in $dMED31^{1/1}$, these abdominal defects were likely the result of abnormal maternal and zygotic dMED31 mRNA production. A mutation in the Mediator subunit dMED13 also caused segmentation defects and this mutant enhanced the *dMED31* mutant maternal effect phenotype. Therefore, our data indicate that the Mediator complex directs zygotic gene expression upon egg deposition to establish cell fate in the embryonic blastoderm.

In order to accomplish cell fate determination, cells gain a transcriptional poised state during early embryogenesis that is maintained throughout development and requires many *cis* and *trans* acting factors that modulate the chromatin environment of the genes involved. In *D. melanogaster*, cell identity along the A/P is established in the blastoderm stage when the pair-rule genes are expressed. A/P polarity is controlled by the maternal morphogenes *cad*, *nos*, *bcd* and *hb* whose activity results in the spatio-temporal expression of the gap genes *gt*, *kni*, *tll* and *Kr* (Hulskamp et al., 1990; Kraut and Levine, 1991; Rivera-Pomar and Jackle, 1996). These gap genes are the first genes expressed along the A/P axis and encode transcription factors that in turn govern patterned expression of the pair-rule genes. Pair-rule gene expression occurs in distinct stripes and is accompanied by cellularization. Thus when cellularization takes place, large clusters of cells gain an imprint that defines the primordial segments. Cell identity is fine tuned when expression of the segment polarity and Hox genes is activated (Sanson, 2001). Although this cascade of maternal, gap, pair-rule and segment polarity genes is well studied, much remains unknown how the maternal morphogens regulate RNAPII activity at their cognate promoters in order to establish regional domains of gap gene expression.

Because segmentation defects in escaper flies derived from $dMED31^{1/1}$ mothers were restricted to the abdomen, it is possible that the bithorax complex (BX-C) is abnormally expressed. This complex contains the homeotic genes *Ultrabithorax*, *abdominal A* and *Abdominal B*, which control the identity of the posterior two-thirds of the fly (Maeda and Karch, 2006). Mutations in *hb*, *Kr*, *tll* and *kni* affect expression of BX-C and result in homeotic transformation (Nauber et al., 1988; Shimell et al., 1994; Casares and Sanchez-Herrero, 1995). We did not observe complete homeotic transformations of entire

parasegments, suggesting an indirect effect of *dMED31*^{1} on Hox activation. Since segment identity is established during early embryogenesis, this implies that only groups of cells and not whole primordial segments gained abnormal imprinting. Regional errors in cellular imprinting are supported by the variety of the abdominal defects we observed in adult flies. Moreover, defects in embryogenesis were accompanied by cell loss at the embryonic poles and aberrant migratory behavior of cells upon gastrulation, processes which occur prior to the activation of the segment polarity and the Hox genes. Finally, early developmental defects coincided with abnormal expression of the gap genes *kni* and *Kr* and subsequently the pair-rule genes *ftz* and *eve*. These genes are expressed prior to Hox gene expression and are required for activation/repression of the Hox cluster. Although it is possible that the abdominal region is preferentially sensitive for a mutation in *dMED31*, it is more likely that random defects during formation of the abdomen are tolerated, whereas defects in other regions of the embryo are incompatible with adult viability and these adults never eclose.

dMED31 and Mediator functioning

Several intriguing questions remain: why is the embryonic phenotype so variable (>90% of the mutant embryos die, while a small percentage of embryos is able to reach the adult stage), why are mainly embryos affected by a mutation in *dMED31* and what is the primary embryonic defect caused by a mutation in *dMED31*? Answers to these questions can be derived from studies of the Mediator in yeast in combination with our data. The budding yeast MED31 protein is part of the Mediator transcription initiation complex (Bourbon et al., 2004; Guglielmi et al., 2004; Linder and Gustafsson, 2004). Although a mutation in yeast *MED31* affects gene expression (van de Peppel et al., 2005), mutants displayed no sensitivity to transcriptional inhibition by 6-azauracil and *MED31* was not essential for growth (Fan et al., 1996; Malagon et al., 2004). However, yeast *MED31* mutants have a synthetic growth defective phenotype when combined with mutations in genes encoding for the two largest subunits of RNAPII (*RPB1*, *RPB2*) and the transcription initiation factors *TFIIB* and *TFIIS* (Fan et al., 1996; Malagon et al., 2004, 2006). Like in yeast, depletion of *dMED31* in *Drosophila* SL2 cells by RNAi did not interfere with the Mediator composition and no growth alterations were reported (Gu et al., 2002). Thus, *Drosophila* MED31, like yeast MED31, might not be essential for RNAPII activity per se, but could be an auxiliary factor involved in the signaling between specific transcription factors and the RNAPII machinery (Park et al., 2001). Together, these findings and our data suggest that dMED31 is not required for transcription in general, but is merely required for the fine tuning of transcription of specific genes.

Largely, based on studies in yeast, it was proposed that the Mediator functions as a platform that allows rapid regulation of transcription at (re)initiation (Asturias, 2004). Fast regulation and (re)initiation of transcription might be key during the interphase periods of the final syncytial cell cycles when zygotic transcription is initiated, while such large scale, strict and "fast" control over transcription would not be essential during subsequent stages of development and thus may explain why dMED31 function is essential during early embryogenesis. The observation that a small percentage of embryos derived from *dMED31*$^{1/1}$ mothers was able to develop into an adult, while the majority of the embryos displayed severe defects during embryogenesis, might also be attributed to such auxiliary function(s) of the dMED31 protein. Minor differences in dMED31 protein levels, due to the hypomorphic *dMED31*^{1} allele, may result in subtle changes in the expression of the gap and pair-rule genes and allow embryos to progress throughout embryogenesis, but with the formation of segmentation defects. On the other hand, in the majority of embryos, more severe changes in gap and pair-rule patterning occur, which results in embryonic death.

In summary, we demonstrate that *dMED31* is essential to establish regional domains of expression of cell fate determinants *kni*, *Kr*, *ftz* and *eve*. mRNA expression of *dMED31* peaks exactly between maternal morphogen and gap gene expression and it was demonstrated that the Mediator complex is able to bind to several maternal transcription factors (Park et al., 2001). Together this indicates that the Mediator complex constitutes an interface between the maternal morphogens and the RNAPII machinery to guide zygotic gene expression of cell fate determinants that specify primordial segment identity. These findings provide the first *in vivo* evidence for a role of the Mediator complex in establishing cell fate during early embryogenesis and since MED31 resembles one of the most conserved subunits within the Mediator complex (Linder and Gustafsson, 2004; Blazek et al., 2005) this protein could serve a crucial role in the control of RNAPII activity during early developmental processes in all higher eukaryotes.

Acknowledgments

We wish to thank H. Jäckle and P. Carrera (Göttingen, Germany) for sharing unpublished antisera against hb, Kr and kni; A. Ephrussi (Heidelberg, Germany) for providing a collection of female sterile mutants; L. Fradkin (Leiden, The Netherlands) for providing the pCasper4-Pme1 plasmid; and P. Gergen (Stony Brook, USA) for providing plasmids to generate *in situ* probes against *eve*, *ftz*, *Kr*, *kni* and *en*. Furthermore, we also thank H.H. Kampinga for discussion and critical reading of this manuscript. This work was financed with a VIDI grant awarded to O.C.M. Sibon by the Netherlands Organization for Scientific Research (NWO;971-36-400).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.11.019.

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