Physiological Implications of impaired de novo Coenzyme A Biosynthesis in Drosophila melanogaster

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CHAPTER 3.

Establishment of cell fate during early Drosophila embryogenesis requires transcriptional Mediator subunit dMED31

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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 knirps and Krüppel and the pair-rule genes fust 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 D. melanogaster.

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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\textsuperscript{1-5} which was first identified in budding yeast\textsuperscript{6} and consists of four large modules; head, middle, tail and a Srb8-11 module\textsuperscript{4,7-9}.

Support for a specific role during development of Mediator subunits is provided by several studies in Drosophila melanogaster\textsuperscript{10-15} and Caenorhabditis elegans\textsuperscript{16-19}. 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\textsuperscript{10} and its presence in the Mediator complex was confirmed in purified complexes from embryos and cells\textsuperscript{20,21}. 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)\textsuperscript{20}. Moreover the Mediator complex is required for in vitro transcription from 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\textsuperscript{w}\textsuperscript{1118} line was used for wild-type preparations. The E709 stock was a generous gift of A. Ephrussi (Heidelberg, Germany). The dMED1\textsuperscript{7} and dMED13\textsuperscript{1} P element insertion lines are previously described\textsuperscript{10} and were obtained from the Bloomington stock centre (Indiana University, USA). P element excision lines were generated with the Δ2-3 transposase\textsuperscript{22}. From a collection of approximately 75 individual excision lines a back to wild-type allele (dMED31\textsuperscript{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 were performed to recover the 3’ and 5’ flanking genomic sequences of the pLacW insertion site\textsuperscript{23}. 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 were 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
Cell fate determination requires dMED31

together with approximately 250 bp of genomic DNA flanking the polyadenylation site was PCR amplified with primer pair 5′-GAAAGATCCGCGGATACATTTAGGGTAG-3′ and 5′-AGCGAATTCTCTGGTGAATTCC-3′, cut with BamHI and EcoRI 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

**Assessment of embryonic viability and abdominal defects:** To assay embryonic viability embryos were collected (0-6 h) on apple juice plates, 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 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+/− mothers crossed with wild-type males were used or embryos deposited by wild-type females crossed with wild-type males. Isolation, fixation and immunolabelling of embryos was performed as described. 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-anti-tailless (1:200, #812) obtained via the Asian Distribution Centre for Segmentation Antibodies, rabbit anti-hunchback (1:1000), rabbit anti-Krüppel (1:500) and rabbit anti-Kr (1:50) (generously provided by H. Jäckle and P. Carrera, Götingen, Germany) and concentrated supernatants of mouse anti-eve-skipped (1:5) (3C10 developed by C. Goodman) and mouse anti-dorsal (1:5) (7A4 developed by R. Steward) obtained from the Developmental 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 ug/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+/− mothers crossed with either dMED31+/+ or dMED31+/− males were pooled and compared to embryos deposited by wild-type mothers crossed with wild-type males. Hybridizations were essentially carried out as described. Plasmids for probe production against Krl (pT:Krl)24, kni (pcJ15)29, eve (p48-X1.4)30, ftz (pT-ftz)31 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* transcripion 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 represents 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), see below) as a candidate for further investigation. Embryos deposited by homozygous females crossed with wild-type males (further referred to as mutant embryos) rarely hatched (2.1%) compared to embryos deposited by wild-type females (88.2%) further referred to as wild-type embryos (*Table 1*). Unless otherwise specified, all studies in this manuscript were performed with embryos derived from females that were crossed with wild-type males. In the mutant, the P element was mapped within the 5′-UTR of CG1057 (*Fig. 1A*) and its 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. SOH1 is also known as TRAP18, but recently unified nomenclature designated the protein MED31, therefore the mutant will be referred to as dMED31. 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<sup>1/1</sup> 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 does 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<sup>1/1</sup> females, mutant females also displayed fecundity defects and cohorts of 10 dMED31<sup>1/1</sup> females produced only 7.4 ± 1.5 eggs/20 h (p < 0.0001) compared to 46.0 ± 3.3 eggs deposited by wild-type females (Table 1). In order to confirm linkage between the observed mutant phenotype and the P element insertion, dMED31<sup>1/1</sup> was placed over the deficiency Df(3R)Z1,ry*, in which at least 82 genes surrounding the dMED31 locus are deleted. Transheterozygous dMED31<sup>1/1</sup>/Df(3R)Z1,ry* females showed more severe fecundity (p < 0.05) defects compared to dMED31<sup>1/1</sup> females (Table 1). A small percentage of embryos derived from dMED31<sup>1/1</sup> 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<sup>1/1</sup>/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<sup>1/1</sup> allele is hypomorphic. Next, we restored the dMED31<sup>1/1</sup> insertion allele by remobilizing the P element<sup>22</sup>. When we placed dMED31<sup>1/1</sup> over a precise excision allele dMED31<sup>1/21</sup>, the hatching rate of embryos deposited by dMED31<sup>1/21</sup> mothers was restored to 91.6%

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**Figure 1. A mutation in dMED31 disrupts 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<sup>1</sup> 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<sup>3</sup>. 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.
Cell fate determination requires dMED31 (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.

<table>
<thead>
<tr>
<th>genotype</th>
<th>hatching rate (%)</th>
<th>fecundity (egg/20 h ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>y1w1118</td>
<td>88.2 (n=1005)</td>
<td>46.0 ± 3.3 (n=45 cohorts)</td>
</tr>
<tr>
<td>dMED311/1</td>
<td>2.1 (n=574)</td>
<td>7.4 ± 1.5 (n=42 cohorts)</td>
</tr>
<tr>
<td>dMED311/Df(3R)Z1,ry*</td>
<td>3.2 (n=632)</td>
<td>3.2 ± 0.4 (n=51 cohorts)</td>
</tr>
<tr>
<td>dMED311/21</td>
<td>91.6 (n=1287)</td>
<td>ND</td>
</tr>
<tr>
<td>P[dMED31];dMED311/1</td>
<td>57.9 (n=401)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*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.
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 wild-type 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 wild-type 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 towards 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) were 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^{1/1}/Df(3R)Z1,ry* or dMED31^{1/1} females crossed with dMED31^{1/1} males. The majority of the embryos deposited by dMED31^{1/1}/Df(3R)Z1,ry* or dMED31^{1/1} mothers (independent of the paternal genotype) displayed

← Figure 2. Mitotic domains associated with migrating cells during gastrulation are mislocalized or absent in dMED31 mutant embryos.

Embryos deposited by wild-type (A-E) and dMED31^{1/1} (F-J) females crossed with wild-type 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) Wild-type 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), posterior midgut rudiment (pmg) are proliferating and the parasegments become visible. (E) The parasemental 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 result in morphologically abnormal embryos. Note that especially cells associated with the migrating polar plate behave abnormal and results in severe deformations at the posterior-dorsal region of the embryo. (K-P) Wild-type 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 M) During st.4-5 of embryonic development the pole cells are formed at the posterior pole of the embryo (arrow). (L) In dMED31 mutant embryos pole cell formation is normal (arrow). (N) but during gastrulation cells associated with the anterior and posterior pole start to delaminate towards the interior of the embryo (arrowheads) and abnormal cell ingestion 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 results in cell loss (asterisks). Scale bars: 20 µm (K-L), 150 µm (M-P).
severe morphological defects beyond st.8 (estimated >90%). Thus, a mutation in the \textit{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, \textit{in vitro} experiments demonstrated that the \textit{Drosophila} Mediator complex is able to bind developmental transcription factors and is essential for the \textit{in vitro} transcription from promoters regulated by these transcription factors\textsuperscript{20}. Moreover, immunoprecipitation of embryonic nuclear extracts with an antibody directed against dMED31 abolished Mediator composition and affected \textit{in vitro} RNA transcription, suggesting that the Mediator complex is required to control gene transcription during early development and requires the dMED31 subunit\textsuperscript{20}. To test this suggestion we first analyzed expression profiles of all Mediator genes using available data sets (www.fruitfly.org and ref. 32). These data show that the entire Mediator complex is maternally supplied to the embryo and is highly expressed prior to gastrulation (see \textit{supplementary Fig. S1}), 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 \textit{supplementary Fig. S1}), suggesting a specific function of the Mediator complex at this specific moment.

Upon egg deposition maternally contributed morphogen gradients of genes such as; \textit{dl}, \textit{cad}, \textit{nos}, \textit{bcd} or \textit{hb} define the embryo’s polarity, the dorsal-ventral (D/V) axis and the A/P axis\textsuperscript{33}. 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 \textit{dl} and \textit{hb} was unaffected in embryos deposited by \textit{dMED31\textsuperscript{1/1}} mothers (Fig. 3E).

During wild-type embryogenesis \textit{cad}, \textit{nos}, \textit{bcd} and \textit{hb} regulate expression of the gap genes \textit{tailless} (\textit{tll}), \textit{giant} (\textit{gt}), \textit{knirps} (\textit{kni}) and \textit{Kr}\textsuperscript{34,35}. dMED31 expression peaks before high expression levels of the gap genes (see \textit{supplementary Fig. S1}) which coincided with the timing of the morphological abnormalities observed in \textit{dMED31} mutant embryos (Fig. 2). These data suggest that gap gene expression may be affected in \textit{dMED31} mutant embryos. To investigate whether \textit{dMED31\textsuperscript{1/1}} indeed affects embryonic gap gene expression we analyzed mRNA and protein distribution of the gap genes \textit{kni} and \textit{Kr} (Fig. 3A). Early mRNA expression of these genes can be observed at the anterior tip and as a band in the centre of wild-type embryos. Furthermore, \textit{Kr} expression is also strong at the posterior end of wild-type 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 \textit{dMED31\textsuperscript{1/1}} females \textit{kni} and \textit{Kr} mRNA expression during st.4-5 is much lower compared to wild-type embryos (Fig. 3A). Moreover, abnormal mRNA and protein expression patterns of these two genes was observed during early stages of embryogenesis. Interestingly, the polar expression of \textit{kni} and \textit{Kr} is hardly detectable or even absent. \textit{dMED31\textsuperscript{1/1}} embryos also displayed abnormal patches of \textit{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 wild-type Kr expression and high Kr expression could also be found in cells outside the central band. Overall 44.6% of dMED31/1 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 pathway36-38. Because dMED31 mutant embryos show terminal defects we analyzed protein expression of tll, whose activity is required to specify cell fates at the embryonic poles39. Protein expression of tll was unaffected in embryos deposited by dMED31/1 females compared to wild-type embryos. 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 mothers, posterior located tll expressing cells were missing (arrows), which was 3.1% in wild-type 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 wild-type 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 mothers the mRNA expression of ftz is lower and abnormal compared to wild-type embryos (Figs. 3B,D). It should be noted that abnormalities in ftz expression are mainly observed during stage 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 wild-type embryos. Wild-type 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 was most obvious in later stages (stage 6-8) (Fig. 3C, data not shown). Based on these observations we conclude that in dMED31/1 mutant embryos expression of eve and ftz is affected. Although at this point we can not 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 mutant embryos due to severe defects during gastrulation. Thus although, the morphological abnormalities (Fig. 2) are uniform and observed in >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 show that the dMED31 subunit is required to establish domains of gap and pair-rule gene expression along the A/P axis during embryogenesis.
Cell fate determination requires dMED31
Wild-type embryos (deposited by wild-type females crossed with wild-type males) and mutant embryos (deposited by $dMED3^{1/+}$ females crossed with pooled $dMED3^{1/1}$ and $dMED3^{1/+}$ 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 wild-type 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 $dMED3^{1/1}$ 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 wild-type (brackets). In wild-type 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 $dMED3^{1/1}$ and wild-type mothers. In wild-type 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 wild-type pattern. However, ftz expression is consistently lower compared to wild-type embryos and becomes abnormal during gastrulation (st.7). Eve mRNA expression is only marginally different in embryos deposited by $dMED3^{1/1}$ compared to wild-type expression (see D), but immunolabellings 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 wild-type embryos, the tll protein is expressed at the posterior cap and as a horseshoe-shaped stripe in the anterior domain. 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 towards the interior of the embryo (see Fig. 2) and in 28.0% ($n=82$) of the embryos deposited by $dMED3^{1/1}$ mothers, posterior located tll expressing cells were missing (arrows), which was 3.1% in wild-type 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 criteria used to score $n$ embryos (depicted at the top of each histogram) for abnormal expression ($p$ values as determined by $\chi^2$ analysis).

(E) Protein analysis of the maternal morphogens dl and hb revealed no differences in expression and localization between embryos deposited by $dMED3^{1/1}$ females and embryos deposited by wild-type 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 (a’ represents a single scan of a”). In the images anterior is to the left and asterisks mark areas of cell loss.

**Mutations in Mediator subunits $dMED31$ and $dMED13$ abrogate abdominal segmentation**

During our analysis of the embryonic hatching rate of eggs deposited by $dMED3^{1/1}$ 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 $dMED3^{1/1}$ females were crossed with wild-type males 33.7% of the cohort of rare escapers that reached the adult stage developed segmentation defects, while this percentage was 45.6% when $dMED3^{1/1}$ females were crossed with $dMED3^{1/1}$ 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
Cell fate determination requires dMED31

dMED31/Df(3R)Z1,ry* or dMED311/1 adults derived from heterozygous parents did not develop significant abdominal deformations (Table 2). Together, this indicates that abnormal maternally supplied dMED31 result in segmentation defects and this effect is enhanced due to aberrant zygotic expression of dMED31. Furthermore, since adults produced by dMED311/+ mothers did not develop segmentation defects (Table 2), the dMED311 allele is recessive.

Figure 4. dMED31 mutants develop abdominal deformations.
Abdominal segmentation was investigated in wild-type and dMED311/2 adult flies derived from homozygous mothers. (A-C) Dorsal, (D-E) ventral and (F-G) lateral views of the abdomen from wild-type (A, D, F) and dMED311/2 (B-C, E, G) females. (A) Wild-type flies have a distinctive pattern of tergites (T1-6). dMED311/2 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 wild-type females displays a distinctive pattern of sternites (S2-7). (E) dMED311/2 female in which 4S formation is abrogated (arrow) (4.1%, n=9). (F) Lateral view of a wild-type haltere (arrowhead). (G) Haltere development is frequently disrupted in dMED31 mutants derived from dMED311/1 mothers (10.6%, n=23). All the above described defects were also found in male mutants. Percentages represent the percentage of total and n indicates the amount of flies with pigment,

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

<table>
<thead>
<tr>
<th>crossings (genotype female X male)</th>
<th>offspring</th>
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<th>n</th>
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<tr>
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<td>2.5</td>
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<tr>
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<td>dMED311/dMED31</td>
<td>15.1</td>
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The total percentage of abdominal defects of n flies in the progeny derived from flies of indicated genotype is shown.
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 specification controlled by the Hox genes proboscipedia and Sex combs reduced during larval development. 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. Since both mutations are lethal we investigated abdominal segmentation in heterozygous mutants. Heterozygous dMED17 mutants displayed normal segmentation (not shown), however, when dMED13+/ females were crossed with wild-type males 22.7% of the dMED13/+ adult progeny had segmentation defects, while 9.8% of the +/+ offspring had defects (Table 2). This indicates that dMED13 is a dominant modifier of segmentation in Drosophila. Most likely abnormal maternal deposition of dMED13 causes segmentation defects in the +/+ offspring and these defects are enhanced by aberrant zygotic expression of dMED13 in the dMED13/+ progeny.

Next we analyzed whether the dMED31 allele could enhance the dominant dMED13 phenotype. When dMED31/ females were crossed with dMED13/TM3 males, 54.7% of the dMED31/dMED13 adults had abdominal defects (Table 2), demonstrating that zygotic expression of the dMED13 allele enhanced the dMED31 phenotype ($\chi^2$ p < 0.006 compared to the progeny from dMED31/ females crossed to wild-type males). However, no increase in segmentation defects was observed in the progeny from dMED13/dMED31 mothers compared to defects observed in progeny from dMED13/+ mothers (Table 2), demonstrating that dMED31 is not an enhancer of dMED13 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/embryos, 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, till and Kr. 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 embryos reach st.10 and expression of the segment polarity and Hox genes is activated by the pair-rule genes and a subset of the gap genes. 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 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. Mutations in hb, Kr, till and kni affect expression of BX-C and results in homeotic transformation. We did not observe complete homeotic transformations of entire parasegments, suggesting an indirect effect of dMED31 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 is 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 fitz 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. Although a mutation in yeast MED31 affects gene expression, mutants displayed no sensitivity to transcriptional inhibition by 6-azauracil and MED31 was not essential for growth. 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. 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. 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. 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. 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 \textit{dMED31/1} 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 \textit{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 occurs, which results in embryonic death.

In summary, we demonstrate that \textit{dMED31} is essential to establish regional domains of expression of cell fate determinants \textit{kni}, \textit{Kr}, \textit{ftz} and \textit{eve}. mRNA expression of \textit{dMED31} (and other Mediator genes) 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. Together this indicates that the Mediator complex constitutes an interface between the maternal mophogens and the RNAPII machinery to guide zygotic gene expression of cell fate determinants that specify primordial segment identity. These findings provide the first \textit{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, this protein could serve a crucial role in the control of RNAPII activity during early developmental processes in all higher eukaryotes.

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LITERATURE CITED


CHAPTER 3
