The Many Facets of bicoid Gradient Formation in Drosophila

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2017

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):

Total number of authors:
1

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The Many Facets of \textit{bicoid} Gradient Formation in \textit{Drosophila}

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LUND UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of medicine, Lund University, Sweden.
To be defended at the lecture hall ‘Segerfalksalen’ at BMC, Sölvegatan 19, Lund
on December 5\textsuperscript{th} 2017 at 13:00.

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Abstract

In *Drosophila*, the Bicoid protein serves as paradigm as the first identified morphogen whose concentration gradient provides the initial positional information in the anterior half of the embryo. There, it differentially activates segmentation genes, in particular the gap genes. Insofar, there are two mainly prevailing models to explain how the Bicoid protein concentration gradient forms: 1) the SDD (Synthesis, Diffusion and Degradation) model proposing that the bicoid mRNA is located at the anterior pole of the embryo at all times. The mRNA then serves as a source for translation of the Bicoid protein, followed by diffusion of the protein to the posterior, combined with uniform degradation. 2) the ARTS (Active RNA Transport and Synthesis) model proposing that the mRNA is transported at the cortex along microtubules to form the mRNA gradient, which serves as template for the production of Bicoid. Hence, there are clear differences between the SDD and the ARTS model.

To corroborate the ARTS model, we used several approaches: 1) we investigated on the transport mechanism of bicoid mRNA. To this end, we detected a cortical microtubular network that was present in the anterior half of the early *Drosophila* embryos which was only active during metaphase and early anaphase. We found that αTubulin67C is crucial for establishing the cortical microtubular network and that cortical bicoid mRNA transport is compromised in αTubulin67C mutants. We defined a motor protein, nonclaret disjunctional (ncd) to be a critical motor for bicoid mRNA transport and demonstrated that ncd interacts genetically with αTubulin67C. This data suggested that ncd required αTubulin67C for cortical bicoid mRNA transport, also demonstrated by colocalization of αTubulin67C with Ncd. 2) we chose one special fly stock that expresses 3 times more Bicoid and treated the embryos with hypoxia to challenge the validity of the SDD model. Our data showed that under hypoxic conditions, the Bicoid protein did not move into the interior, but rather moved along the cortex, even during long exposures. 3) Combining hypoxia with drugs that disturb the formation of microtubules, we could observe interior movement of Bicoid, while the mRNA strictly remained at the tip. When actin was compromised, little cortical Bicoid movement was observed. This data suggested that Bicoid requires an intact cytoarchitecture for cortical movement. Finally, we revealed several factors that played distinct roles in bicoid mRNA gradient formation, including trans-Golgi proteins, the poly(A) polymerase Wispy, CyclinB and egg-activation genes.

Apart from studying the mechanism of bicoid gradient transport, we explored the expression patterns of bicoid-downstream genes in *Bactrocera dorsalis*, which is the oriental fruit fly with high relatedness to *Drosophila*, however lacking bicoid. When comparing the segmentation gene expression patterns between *Drosophila* and *Bactrocera*, bicoid downstream genes showed a strong shift of expression towards the posterior suggesting that the positioning of the segmental anlagen along the anterior-posterior axis changed during evolution.

Key words: *Drosophila*, bicoid, gradient formation, ARTS model, cortical microtubule, drug,
The Many Facets of *bicoid*
Gradient Formation in *Drosophila*

Xiaoli Cai
Coverphoto: Analysis of Bicoid movement in Drosophila embryos exposed to hypoxia and different drugs, and monitored using heat-maps.

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Faculty of Medicine
Department of Experimental Medical Science

Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:178
ISBN 978-91-7619-560-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund 2017
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ABSTRACT

In Drosophila, the Bicoid protein serves as paradigm as the first identified morphogen, whose concentration gradient provides the initial positional information in the anterior half of the embryo. There, it differentially activates segmentation genes, in particular the gap genes. Insofar, there are two mainly prevailing models to explain how the Bicoid protein concentration gradient forms: 1) the SDD (Synthesis, Diffusion and Degradation) model proposing that the bicoid mRNA is located at the anterior pole of the embryo at all times. The mRNA then serves as a source for translation of the Bicoid protein, followed by diffusion of the protein to the posterior, combined with uniform degradation. 2) the ARTS (Active RNA Transport and Synthesis) model proposing that the mRNA is transported at the cortex along microtubules to form the mRNA gradient, which serves as template for the production of Bicoid. Hence, there are clear differences between the SDD and the ARTS model.

To corroborate the ARTS model, we used several approaches: 1) we investigated on the transport mechanism of bicoid mRNA. To this end, we detected a cortical microtubular network that was present in the anterior half of the early Drosophila embryos, which was only active during metaphase and early anaphase. We found that αTubulin67C is crucial for establishing the cortical microtubular network and that cortical bicoid mRNA transport is compromised in αTubulin67C mutants. We defined a motor protein, nonclaret disjunctional (ncd) to be a critical motor for bicoid mRNA transport and demonstrated that ncd interacts genetically with αTubulin67C. This data suggested that ncd required αTubulin67C for cortical bicoid mRNA transport, also demonstrated by colocalization of αTubulin67C with Ncd. 2) we chose one special fly stock that expresses 3 times more Bicoid and treated the embryos with hypoxia to challenge the validity of the SDD model. Our data showed that under hypoxic conditions, the Bicoid protein did not move into the interior, but rather moved along the cortex, even during long exposures. 3) Combining hypoxia with drugs that disturb the formation of microtubules, we could observe interior movement of Bicoid, while the mRNA strictly remained at the tip. When actin was compromised, little cortical Bicoid movement was observed. This data suggested that Bicoid requires an intact cytoarchitecture for cortical movement. Finally, we revealed several factors that played distinct roles in
bicoid mRNA gradient formation, including trans-Golgi proteins, the poly(A) polymerase Wispy, CyclinB and egg-activation genes.

Apart from studying the mechanism of bicoid gradient transport, we explored the expression patterns of bicoid-downstream genes in Bactrocera dorsalis, which is the oriental fruit fly with high relatedness to Drosophila, however lacking bicoid. When comparing the segmentation gene expression patterns between Drosophila and Bactrocera, bicoid downstream genes showed a strong shift of expression towards the posterior suggesting that the positioning of the segmental anlage along the anterior-posterior axis changed during evolution.
LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text with their Roman numerals.


III Cai, X., Fahmy, K. and Baumgartner, S. Genetic analysis of factors influencing bicoid gradient formation in Drosophila. (manuscript: deleted as per Lund University library request)

ABBREVIATIONS

aa  amino acid
aMTOC  acentriolar microtubule organizing center
AP  anterior-to-posterior
ast  asterless
bcd  bicoid
bp  base pair
cnn  centrosomin
chb  chromosome bow
CLASP  cytoplasmic linker-associated protein
CNS  central nervous system
dpp  decapentaplegie
DV  dorsal-to-ventral
ems  empty spiracles
en  engrailed
eve  even-skipped
FGF  fibroblast growth factor
hb  hunchback
Kr  Krüppel
MT  microtubule
MTOC  microtubule organizing center
nos  nanos
nt  nucleotide
osk  oskar
otd  orthodenticle
PA  posterior-to-anterior
PAP  Poly(A) polymerase
PCM  pericentriolar material
PEST  proline (P), glutamic acid (E), serine (S) and threonine (T)
RNP  ribonuclear protein
SID  self-inhibitory domain
spd-2  spindle defective 2
TGN  trans-Golgi network
γ-TURC  γ-tubulin ring complex
wg  wingless
1 INTRODUCTION

“Developmental biology deals with the process by which the genes in the fertilized egg control cell behavior in the embryo and so determine its pattern, its form, and much of its behavior… differential gene activity controls development” (Wolpert, 1998).

Mice, frogs, zebrafish, C. elegans or Drosophila melanogaster? Each idealized animal model has its own strength and weakness. Critics have mainly questioned whether those animal models are qualified to represent the rest of the animal species, since the requirements of those animals as study models were subject to the bias of their life cycles and developmental advantage.

Thanks to the pioneering genetic studies of Thomas Hunt Morgan beginning in 1910, the history of using Drosophila as an animal organism in modern biological sciences spans a century. Executed as a pilot study before sequencing the human genome, Drosophila was the first complex animal to have its entire genome sequenced (Adams et al., 2000). Regardless of the different developmental patterns, the highly conserved genes and regulatory mechanisms that Drosophila shares among all the animals draw more scientific attention (Reiter et al., 2001). In Drosophila, there are approximately 15,600 genes within a genome of 144 million base pairs. The genes associated with known diseases that range from the central nervous system to cardiovascular diseases, from cancer to metabolic disorders, share nearly 77% similarity with their human counterparts (Kornberg and Krasnow, 2000; Pandey and Nichols, 2011). For instance, the major components of the Ras signaling pathway in the fly visual system have been found to be highly conserved in mammalian cells (Cagan, 2009; Nagaraj and Banerjee, 2004), which contribute the research of cancer biology. As a model for neurodegenerative diseases, when the CNS of the flies was transfected with Parkinson’s disease-associated genes, flies displayed similar symptoms with those from human Parkinson’s patients (Feany and Bender, 2000; Whitworth, 2011).
1.1 Overview of the *Drosophila* life cycle

The life journey of *Drosophila* begins with the fertilized egg, which undergoes its embryogenesis within the first 24 hours. Afterwards, a first instar larva hatches out of the egg. This larva eats to increase its size during the next 4.5 days, while going through two more instars. At day 5.5, the larva forms the pupa by secreting a pupal case. During the pupal stage, a significant phase called metamorphosis takes place resulting in the formation of the adult structures. The last step is the hatching of the adult fly, which completes the life cycle. The life cycle of *Drosophila* takes 9 days at 25° C and is demonstrated in Fig. 1.

![Life cycle of Drosophila melanogaster](image)

*Fig. 1 Life cycle of Drosophila melanogaster*

1.2 Overview of early *Drosophila* development

The early stage of *Drosophila* embryogenesis is represented by 14 nuclear cycles during the first 3 hours, in the absence of cytokinesis. The first nuclear cycle (nc) occurs after the fusion of the male and female pronuclei, whereby the syncytial cycles are initiated. The first 7 nuclear divisions occur at the center along the anterior-posterior axis. From the 8th division on, the nuclei start to migrate toward the periphery of the embryos. During the period of nc 9, approximately five nuclei move to the posterior pole and become enveloped by cell membranes. These give rise to pole cells that later will become the gametes of the adult. The remaining nuclei distribute along the cortex from nc 10 on, and complete the last 4 divisions. During the 14th nuclear cycle, cell membranes encapsule the nuclei, forming the cellular blastoderm. Fig. 2 shows the cleavage stages of *Drosophila* embryos.

![Fig. 2 Drosophila melanogaster: early embryogenesis](image)

After the first 3 hours and 14 nuclear divisions, the embryo is at the cellular blastoderm stage, which contains approximately 6,000 nuclei at the embryonic surface. Image taken from the book “Principles of Development”, Wolpert et. al., 5th ed. (2015), Sinauer, ISBN 978-0-19-967814-3

1.3 Genes that govern the *Drosophila* body plan

The commitment of *Drosophila* body patterning consists of two steps: specification and determination. The specification of the cell fate depends on signals provided by protein gradients that originate from maternal genes, and can still be influenced in response to other cells. The specified cell fate is transformed into an irreversible determination by zygotic genes, categorized as segmentation genes (gap genes, pair-rule genes, and segment polarity genes), and becomes cell-intrinsic.

1.3.1 Maternal-effect genes

(Marlow, 2010) describes: "Maternal genes are those genes whose products, RNA or protein, are produced or deposited in the oocyte or are present in the fertilized egg or embryo before expression of zygotic genes is initiated." During the early
embryonic stage in *Drosophila*, maternal genes that are dumped from nurse cells to oocytes, are responsible for programming the early embryogenesis, specifically in regards to the formation of the body axes: the AP and DV axes (Fig. 3).

One of the most important maternal genes is *bicoid (bcd)*, the first identified morphogen found in a maternal-effect mutation screen (Frohnhofer et al., 1986). Bcd is known to affect multiple maternal and zygotic genes by initiating expression patterns in relation to the AP axis (Fig. 4).
**Fig. 4 Segmental cascade initiated by maternal genes along the AP axis**

Once the Bcd morphogen provides pattern information, the downstream genes are activated sequentially and spatially. Segments: Lr, labral; hb, preantennal domain (head blob of wingless); hs, preantennal domain (head spot of engrailed); An, antennal; lc, intercalary; Md, mandibular; Mx, maxillary; La, labial; T1-3, thoracic segments; A1-9, abdominal segments. bcd, bicoid; otd, orthodenticle; ems, empty spiracles; bid, bottomhead; hh, hunchback; Kr, Krüppel; kni, knirps; gt, giant; cad, caudal; fz, fushi tarazu; eve, even-skipped; h, hairy.

Image credit: Adapted from Dmitri Papatsenko (https://bspace.berkeley.edu/access/content/user/247388/index.html)

### 1.3.2 bicoid, a maternal effect gene determines the AP axis

*bcd* belongs to the homeobox gene family and stems from an ancestral Hox3 gene. An interspecific comparison between *D. melanogaster* and *D. pseudoobscura* was carried out by (Seeger and Kaufman, 1990) identifying conserved functional domains of *bcd* in *Drosophila*. In the past 30 years, numerous studies on Bcd have been carried out at the molecular, cellular and physical level, all devoted to uncover the DNA and protein structure, and to determine the fundamental roles and the mechanism of Bcd. Many studies have provided experimental data that Bcd was the first true morphogen, playing a role via its protein concentration gradient along the AP axis during *Drosophila* embryonic development (Fig. 5) (Crauk and Dostatni, 2005; Little et al., 2013; Porcher and Dostatni, 2010; Wieschaus, 2016).
In oogenesis, bcd mRNA is synthesized in the nurse cells, then dumped into the oocyte and finally transported to the anterior tip. After egg activation, bcd mRNA is translated into proteins during the syncytial blastoderm. With the positional signal provided from the Bcd protein gradient, the anterior zygotic genes are up- or down-regulated to form the AP axis. Image taken from the book “Developmental Biology”, Gilbert and Barresi, 11th ed. (2016), Sinauer, ISBN 978-1605354705

1.3.3 Analysis of the Bcd sequence and its functional domains

(Berleth et al., 1988) was the first report that determined the sequence of bcd. The study also identified a shorter isoform containing only exons 1 and 4, which arises through alternative splicing. Compared to the length of the current known Bcd (large Bcd), the product of the 1.4 kb isoform was later designated ‘small Bicoid’ (Fig. 6B).
The Bcd protein (494 aa) is divided into 7 independent domains (Fig. 6B). The main functional domain of Bcd, also called the homeo-domain, resides in the amino-terminal part and is responsible for facilitating its morphogenic activity by binding DNA or RNA in a sequence specific manner (Baird-Titus et al., 2006; Rivera-Pomar et al., 1996). Homeo-domains are evolutionarily-conserved domains encompassing exactly 60 amino acids that regulate developmental processes during embryonic pattern formation in many multicellular organisms. Interestingly, the Bcd homeo-domain is considered to be unique. (Baird-Titus et al., 2006; Dave et al., 2000) demonstrated that the homeo-domain of Bcd contains not only a lysine residue at the critical position 50 (K50), but also has an arginine residue at position 54 (R54). With this flexibility, bcd is enabled to re-program its recognition codes corresponding to consensus or non-consensus target-binding sites, and alternates its role as a transcriptional and translational regulator. Further functional and structural studies demonstrated that the helix 3 of Bcd adopts multiple conformations when bound to different targets. Further, it was proposed that the conformational heterogeneity of the homeo-domain could play an important role in admitting Bcd to read its various binding zones (Adhikary et al., 2017; Dave et al., 2000).
Immediately after the homeo-domain, a domain between aa 170 and 203 constitutes a PEST domain, which regulates the degradation of Bcd and results in a half-time of Bcd shorter than 30 min (Driever and Nusslein-Volhard, 1988).

Approximately 80 aa upstream of the carboxy-terminal end of Bcd, an acidic motif was found, which was also identified as the main domain of small Bcd. Studies of (Driever and Nusslein-Volhard, 1989; Struhl et al., 1989) concluded that the acidic domain was not required for mediating the transcriptional activity of Bcd, but to maximize bcd activity.

### 1.3.4 The homeo-domain-less Bicoid protein (small Bicoid)

The small Bicoid (smBcd) lacks the homeo-domain, the PEST domain, the Q- and A-rich domain as well as other domains of unknown function, and is thought to modulate the transcriptional activity of the main Bcd protein (large Bcd) (Zhao et al., 2003). It has been reported that the other important maternal gene osk in Drosophila encodes two protein isoforms, termed long Osk and short Osk (Ephrussi and Lehmann, 1992; Hurd et al., 2016; Markussen et al., 1995; Vanzo et al., 2007). Both isoforms together regulate the downstream genes at the posterior end, and determines the germ cell fate and the posterior polarity. Between the two, the short Osk is part of the germ plasm and is indispensable for the formation of functional primordial germ cells. However, in contrast to Osk, there is sparse structural and functional information on smBcd, because this isoform was largely neglected.

Current studies reveal that smBcd lacks the most important functional domain, the homeo-domain. Moreover, the splice event leads a truncated SID domain (self-inhibitory domain, see Fig. 6B) that contains a particular 10 amino acid stretch identified for modulating the transcriptional activity of large Bcd (Zhao et al., 2002). Interestingly, exon 1 of smBcd ends exactly within the 6 amino acids important for this function. Mutational studies have shown that, by altering the last 4 amino acids of the SID in the large Bcd (located on exon 2 and thus absent in small Bcd), the transcriptional activity of Bcd is lowered by a factor of 100 in CAT-assays (Zhao et al., 2003), indicating a particular function ascribed to smBcd.

### 1.3.5 Large Bcd as a transcription and translation factor

Due to its flexible binding ability to both DNA and RNA, scientists have identified more than 40 zygotic target genes and 66 enhancers that are directly mediated in a Bcd-dependent manner (Chen et al., 2012; Driever and Nusslein-Volhard, 1989; Howard and Struhl, 1990; Kantorovitz et al., 2009; Riddihough and Ish-Horowicz,
Bcd regulates its target genes in both positive and negative ways. One well-studied example is the establishment of the expression pattern of hunchback (hb). hb contains at least 8 Bcd binding sites and highly depends on Bed to maintain its domain of transcriptional activity in the anterior half of the embryo (Driever and Nusslein-Volhard, 1989; Lucas et al., 2013). (Niessing et al., 1999) revealed that the PEST motif of Bcd participates in the translational repression of caudal (cad) through binding to the 3’ untranslated region (3’UTR) of the cad mRNA in the anterior part of the embryo. Furthermore, (Rodel et al., 2013) added that Bcd binds to the cad 3’UTR together with the help of the miR-2 microRNA. (Lasko, 2012) described that the Nanos gradient along the PA axis is achieved through translational-repressing activity of Bcd. Fig. 7 shows the interaction domains between Bcd and its target genes (McGregor, 2005).

**Fig. 7 The functional domains of Bcd**
The homeo-domain is shown in dark blue with the lysine and arginine residues at positions 50 and 54, respectively, and highlighted in yellow. Amino acids involved in cooperative Bcd binding are indicated by arrows. The SID, which can reduce the transcriptional activity of Bcd is shown in cyan. The elf4E-binding domain (shown in purple) blocks the initiation of the translation complex at the 5'-cap of cad transcripts. The PEST domain (shown in black) also contributes to the repression of cad translation as well as in transcriptional activation and Ubiquitin-dependent protein degradation. The glutamine (Q)-rich transcriptional activation domain (shown in green) can be down-regulated by the alanine (A)-rich transcriptional repression domain (shown in red). The C-terminal acidic region (shown in green) is also a transcriptional activation domain. Image from (McGregor, 2005).
1.4 The recent origin of \( bcd \) during insect radiation

As a member of the homeobox genes, \( bcd \) was considered to be as conserved as other homeobox genes in animal evolution. However, \( bcd \) homologs are rarely found in other species, apart from \textit{Drosophila melanogaster}, and were found only in the family of dipterans, such as \textit{Megaselia} (Stauber et al., 1999; Stauber et al., 2000), \textit{Musca} and \textit{Lucilia} (Shaw et al., 2001). On this basis, it was suggested that \( bcd \) evolved recently and was derived from the ancestral \textit{Hox3} gene during insect evolution (Stauber et al., 1999; Stauber et al., 2002). This suggestion is peculiar, leading to questions of how the \( bcd \) gene evolved and adopted its unique function in organizing its patterning activity along the AP axis, and how other insects would execute this task without \( bcd \).

1.5 Two controversial models of Bcd gradient formation

Regardless of its function as a transcriptional or a translational factor, the Bcd protein gradient along the AP axis is an important information source at the early stage of embryonic development. The mechanisms behind the establishment of this concentration gradient have attracted the attention of scientists across many disciplines. At present, there are two opposing, but well investigated models.

1.5.1 The SDD model and its paradox

Since its discovery in 1988, the Bcd gradient has been analyzed extensively by various methods (Ephrussi and St Johnston, 2004). By utilizing various antibodies against the Bcd protein, an anterior exponential gradient reaching > 50% egg length was detected (Driever and Nusslein-Volhard, 1988). To explain the occurrence of the gradient, a simple cellular mechanism was put forward: Bcd is synthesized from a local source of mRNA, followed by diffusion and uniform degradation. This mechanism would generate non-uniform distribution of Bcd along the AP axis to regulate pattern formation (Driever and Nusslein-Volhard, 1988; Fradin, 2017; Gregor et al., 2007) (Fig. 8), also termed the SDD model (synthesis, diffusion and degradation). As one member of the morphogen family, it was reasonable to propose diffusion as the mode of transport of the Bcd (Turing, 1952). In fact, several well-studied morphogen gradients including those of Dpp, Wg and Fgf8, largely satisfy the principle of the diffusion model (Grimm et al., 2010; Kicheva et al., 2007).
Even though the SDD model can be extended to several other morphogens, the model recently encountered a critical challenge. Combining the diffusion equation to estimate the diffusion coefficient ($D$) of Bcd and the framework of SDD model, it was expected that $D$ should be approximately 2 $\mu m^2/s$ to reach a stable concentration along the cortex within 90 minutes. However, results from Fluorescence Recovery After Photobleaching (FRAP) identified that the $D_{\text{FRAP}}$ was stable at approximately 0.3 $\mu m^2/s$, which is at least one order of magnitude lower than expected (Gregor et al., 2008; Gregor et al., 2007). This fact questions the possibility that the gradient could be established so rapidly (i.e. within 90 minutes).

(Abu-Arish et al., 2010; Porcher et al., 2010) measured the Bcd coefficient using Fluorescence Correlation Spectroscopy (FCS) and found that the diffusion coefficient ($D_{\text{FCS}} \sim 7 \mu m^2/s$) was too high to be consistent with the SDD diffusion model. In their studies, $D_{\text{FRAP}}$ was measured as well, and parameters were close to 1 $\mu m^2/s$. (Sigaut et al., 2014) explained the results from both diffusion efficient ($D_{\text{FCS}}$ and $D_{\text{FRAP}}$) by proposing that the results could be compatible, if one considers the interaction of Bcd with slowly-moving binding sites.
Already in 1986, the presence of an mRNA gradient had been shown (Frigerio et al., 1986), adding further to the inconsistencies within the SDD model. However, this report lacked a detailed analysis and hence the results were neglected for at least two decades. However, research has resumed once again in recognition of the importance of the mRNA gradient along the anterior-posterior axis.

1.5.2 The ARTS model

In 2009, (Spirov et al., 2009) published a seminal article demonstrating that the mRNA itself forms a concentration gradient along the embryonic cortex (Fig. 9), in accordance with the previous findings of the existence of a mRNA gradient 3 decades ago (Frigerio et al., 1986). This mRNA gradient decrease exponentially from the anterior pole of the embryo at the syncytial blastoderm stage and at all stages precisely parallels that of the Bcd protein distribution.

![Fig. 9 The bcd mRNA (top) and Bcd protein gradient (bottom).](image)


In order to achieve the rapid transport of the bcd mRNA within the time limitations, active and fast transport of the mRNA along a cortical network of microtubules (MTs) was proposed, a model referred to as the ARTS model (active
RNA transport and synthesis (Spirov et al., 2009). The ARTS model differs dramatically from the SDD model and provides a reasonable explanation for the paradoxical results, that the diffusion of the Bcd protein was too slow by one order of magnitude (Gregor et al., 2007).

1.6 The cornerstones behind the ARTS model

1.6.1 The secondary structure of the bcd 3’UTR and its respective roles

How is the active transport of the bcd mRNA achieved at the molecular level? It is presumed that the bcd mRNA localization is highly dependent on its 3’UTR, which folds into a particular secondary structure showing three arms (Fig. 10). Two arms, domains IV and V, are responsible for the localization of the mRNA by binding to Staufen, a RNA-binding protein (Ferrandon et al., 1994). The third arm, domain III, is responsible for dimerization (Wagner et al., 2004). Approximate 40 nt downstream of the stop codon, a degradation motif was identified (Surdej and Jacobs-Lorena, 1998) providing a tool to regulate the amount of bcd mRNA. The Staufen-mRNA complex, along with other proteins, is thought to bind Dynein or Kinesin, two motor proteins, which allow rapid movement of the complex along microtubules (MTs). In essence, it is this large bcd ribonuclear protein (RNP) complex that migrates along MTs at the cortex of the embryo to form the bcd mRNA gradient.

Fig. 10 Secondary structure of the bcd 3’ UTR
The structure shows the elements for localization, dimerization and degradation. Image from (Brunel and Ehresmann, 2004)
1.6.2 *bcd* mRNA localization in oocytes

In the *Drosophila* oocyte, the process for *bcd* mRNA microtubule-dependent localization was demonstrated (Chang et al., 2011; Khuc Trong et al., 2015; Stephenson and Pokrywka, 1992). In the oocyte, at stage 10b and onwards, the γ-tubulin ring complex (γ-TURC) as the microtubule organizing center (MTOC) assembles microtubular tracts for *bcd* mRNA transportation (Weil et al., 2006). MTs are nucleated or anchored at the cortex and spread from the periphery into the interior. More importantly, cortical MTs follow a decreasing concentration gradient along the cortex, consistent with the distribution of the *bcd* mRNA (Weil et al., 2010). According to their model (Fig. 11) and supported by experimental data, distinct roles of Staufen and Dynein were proposed during late *Drosophila* oogenesis. It was described that both Staufen and Dynein were closely associated with the *bcd* mRNA to form the *bcd* RNP. The *bcd* RNP moved along specialized MTs at the anterior pole, maintained by the cortical actin cytoskeleton. Together, localization of *bcd* mRNA along the embryonic cortex is a complex process including transacting factors (e.g. Staufen), molecular motor proteins (e.g. Kinesin and Dynein) and cytoskeletal components (e.g. microtubules) that remodel extensively during the lifetime of the mRNA.

![Fig. 11 Model for bcd mRNA localization in late oogenesis](image)

MTs (green) are localized by actin (blue), and *bcd* mRNA (red) moves along the MTs with the help of dynein (purple) as a RNP complex. Image from Development 137, 169-176 (2010)

More recent studies suggested that the anchoring of the *bcd* mRNA after being transported to the anterior was not MT-dependent. (Trovisco et al., 2016)
depolymerized microtubules by incubating the embryos in the presence of Colcemid. However, a large percentage of the \( bcd \) mRNA remained stable. In addition, microtubules were stained with the minus-end binding protein Patronin. The result showed that the \( bcd \) mRNA and the MT minus-ends do not overlap, even though both proteins are strongly localized at the anterior pole of the oocyte. Considering all these significant findings, it was proposed that only the delivery of \( bcd \) mRNA towards the anterior pole needed MTs as tracts. However, as for anchoring the \( bcd \) mRNA, MTs would be required to cooperate with other components to specifically anchor the RNA along the anterior cortex.

The mechanisms of \( bcd \) mRNA transport during oocyte development shed promising light on how the Bcd protein gradient formation could occur during early \textit{Drosophila} embryogenesis. Most importantly, however, it should be taken into account that all MT arrays are disassembled into short and un-oriented MT filaments through the late stage of oogenesis (Steinhauer and Kalderon, 2006; Theurkauf and Hawley, 1992; Theurkauf et al., 1992). This means that the embryo after fertilization needs to build up a fresh MT network.

### 1.6.3 Cortical MT network at blastoderm embryos

In contrast to the wealth of information on the \( bcd \) mRNA transport during oogenesis, information in regards to the cortical MT network for \( bcd \) mRNA transportation during embryogenesis was sparse. Most reports focused on detecting how the MTs cooperate with other microfilaments such as actin for the development of the cytoskeleton during the syncytial blastoderm (Karr and Alberts, 1986; McCartney et al., 2001; Rodriguez et al., 2003; Sullivan and Theurkauf, 1995).

(Fahmy et al., 2014) detected a peculiar cortical MT network in the anterior half of early nuclear cycle 1-6 embryos. The study also showed that when the cortical MT network or the motor protein was compromised, e.g. through mutations in the respective gene, \( bcd \) mRNA transport was not established along the cortex anymore.

Since it was shown that the cortical MT network is indispensable to the formation of \( bcd \) mRNA gradient during early embryogenesis, an important question comes up: where are the cortical MT arrays nucleated?
1.7 MT organizing center

The centrosome is the conventional microtubule organizing center (MTOC) in cells and is mainly involved in the process of meiosis and mitosis. MT arrays organized from the centrosome are astral, whose minus-ends are embedded in the centrosome, while the highly dynamic plus-ends stretch outwards.

It has been shown that MT arrays can also be nucleated from centrosome-independent centers, termed acentriolar microtubule organizing centers (aMTOCs) (Baumbach et al., 2015; Moutinho-Pereira et al., 2009). The MT arrays that are assembled by aMTOCs are asymmetric. (Yvon and Wadsworth, 1997) found non-centrosomal acting MTs that emanated from the peripheral lamella of human kidney cells that behaved dynamically at each end. (Khodjakov et al., 2000) deactivated one or two centrosomes, respectively, and still could find largely functional MTs. (Nguyen et al., 2011) showed that in Drosophila neurons, MT arrays were formed independently of the centrosome and did not require centrosomes for maintenance of those MTs. In Drosophila somatic cells, monopolar spindle MTs were assembled with the assistance of aMTOCs, but without recruitment of centrosomal- and chromatin-MTs (Mottier-Pavie et al., 2011). Likewise, in others specialized cell types, a number of aMTOCs have been detected, among these are melanosomes in the pigments cells (Malikov et al., 2004), the plasma membrane of polarized epithelial cells (Reilein and Nelson, 2005), and nuclear envelope in myotubes (Bugnard et al., 2005). However, studies have showed that MT nucleation of aMTOC to a certain degree relies on the known centrosomal pericentriolar material (PCM) with the assistances of Asterless (Asl) serving as a member of mother centrioles. In parallel, Centrosomin (Cnn) and Spindle defective 2 (Spd-2) serve as PCM scaffolding proteins (Baumbach et al., 2015).

1.7.1 Golgi complex as an aMTOC

In addition to the above, numerous reports showed that MT-assembling structures could emanate from Golgi membranes (Chabin-Brion et al., 2001; Sanders and Kaverina, 2015; Stephens, 2012; Zhu and Kaverina, 2013) (Fig. 12). A role of MTs nucleated at the Golgi complex was ascribed to help to maintain the integrity of the Golgi stacks (Miller et al., 2009), and stabilizing the position of the Golgi complex within cells (Derby et al., 2007; Efimov et al., 2007).
Fig. 12 Golgi as an aMTOC.
Golgi complex is as an aMTOC to nuclear MTs via both cis- and trans-golgins. Image from (Stephens, 2012)
Image credit: (Stephens, 2012)

The golgins, a family of large coiled-coil proteins thought to function as tethers are associated with the cytoplasmic surface of the Golgi complex (Nozawa et al., 2005). dGM130 and dGM210 are localized within the cis-Golgi, while the GRIP domain-containing golgins (dGolgin-97, dGolgin-245, dGCC88, and dGCC185, all decorate the surface of the trans-Golgi network (TGN). Most of them have binding sites for the Rab family GTPases for the purpose of anchoring to the Golgi membranes (Sinka et al., 2008; Yadav and Linstedt, 2011), see also Table 1.
Table 1 Golgi binding sites of GTPases

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Localization</th>
<th>GTPases</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGM130</td>
<td>cis-Golgi</td>
<td>Rab1, Rab30</td>
<td></td>
</tr>
<tr>
<td>dGMAP210</td>
<td>cis-Golgi</td>
<td>Rab2</td>
<td>MTs</td>
</tr>
<tr>
<td>dGolgin-97</td>
<td>trans-Golgi</td>
<td>Rab6, Arl1/3</td>
<td></td>
</tr>
<tr>
<td>dGolgin-245</td>
<td>trans-Golgi</td>
<td>Rab2, Arl1/3</td>
<td></td>
</tr>
<tr>
<td>dGCC88</td>
<td>trans-Golgi</td>
<td>Rab6, Arl1/3</td>
<td></td>
</tr>
<tr>
<td>dGCC185</td>
<td>trans-Golgi</td>
<td>Rab2, Arl1</td>
<td>MTs</td>
</tr>
</tbody>
</table>

In nocodazole wash-out hepatic cells, Golgi-dependent MTs are reconstituted and anchored, together with the participation of γ-tubulin (Chabin-Brion et al., 2001). Later, it was shown that a cis-Golgi MT binding protein, GMAP-210 was involved in tethering γ-tubulin to the Golgi membranes in mammalian cells (Rios et al., 2004). GM130 captures nascent MTs directly with the exception of GMAP-210 (Wei et al., 2015). Following up with prior research, (Efimov et al., 2007; Goud and Gleeson, 2010) subsequently demonstrated that the TGN plays an important role in both MT recruitment and stabilization. They found that on the surface of TGN, a MT plus-end binding protein, cytoplasmic linker-associated protein (CLASP), is trapped to the TGN and which recruits the MTs, with assistance of dGCC185.

Apart from the Golgi complex as a potent aMTOC in Drosophila embryos, the MT minus-end protein, Patronin, was reported to be involved in capping MTs. This finding by (Goodwin and Vale, 2010) showed that purified Patronin was sufficient to cap and protect the minus-end of MTs against depolymerization in Drosophila S2 cells. In Patronin-deficient cells, free MT arrays were found moving through the cytoplasm. (Nashchekin et al., 2016; Takeichi and Toya, 2016) elucidated that in Drosophila oocytes, Patronin was located along the cortex by interacting with a cortical anchor, named ‘Short stop’, for the recruitment of noncentrosomal MTs to establish the AP axis.

1.8 Other factors regulating bcd activity

1.8.1 Regulation of poly(A) tail length

When comparing the bcd mRNA and protein patterns, it is obvious that the protein gradient reaches more posterior areas than the mRNA gradient. While the latter could be due to lack of sensitivity to detect a longer mRNA gradient, it does not exclude the possibility that there is post-transcriptional control involved that allows to stretch the gradient further to the posterior.
Previous reports have shown that the translational efficiency of Bcd strongly depends on the length of the poly(A) tail of the bcd mRNA (Salles et al., 1994), see also Fig. 13.

![Fig. 13 poly(A) tail length analysis](image)

Tests show that the poly(A) tail changes with time and is highest at the time of nc 14 Image from (Salles et al., 1994).

The Drosophila genome harbors two poly(A) polymerase (PAP), wispy and pap2. Overexpression of PAP2 level leads to a substantial increase of bcd protein concentration (Juge et al., 2002). In the oocyte, during mid to late oogenesis, wispy assists gurken mRNA localization at the dorsal-anterior corner through its role of polyadenylation (Derrick and Weil, 2017; Norvell et al., 2015). Before egg activation, the poly(A) tail length of bcd was short, preventing translation of Bcd (Eichhorn et al., 2016). Moreover, embryos from wispy mothers failed to go through oocyte maturation due to defects with MT-based events, which are essential for bcd, nos or osk mRNA localization to either pole (Brent et al., 2000). In summary, both PAP2 and wispy are required sequentially in cytoplasmic polyadenylation of Drosophila oogenesis and in the early developmental stage. PAP2 is responsible during the mid-oogenesis, while wispy is active from the late oogenesis to early embryogenesis (Benoit et al., 2008).

1.8.2 Cyclins

In syncytial Drosophila embryos, the “wait-until-ready” mechanism of spindle assembly checkpoint (SAC) coordinating the mitotic events in slower-dividing nuclei from nc 13 on appears inappropriate and dispensable (Yuan and O'Farrell,
For this reason, a cyclin-based intrinsic timer was adopted. There are three mitotic cyclins (A, B, and B3) that function and timely control the phase-phase transition in syncytial embryos. The sequential degradation of CyclinA, CyclinB and CyclinB3 is responsible for the timing and coordination of mitotic events. CyclinA is degraded prior to the metaphase-anaphase transition and is responsible for sister chromosome disjunction, while CyclinB3 is degraded during anaphase and facilitates the restoration of the interphase nucleus. In contrast, the degradation of CyclinB contributes to the transition from metaphase to anaphase (Parry and O'Farrell, 2001; Yuan and O'Farrell, 2015). Interestingly, CyclinB is a target of Wispy (Cui et al., 2013). Due to the fact that the activity of the cortical MT threads for bcd mRNA transport are cell-cycle-dependent and are detected during metaphase and early anaphase only (Fahmy et al., 2014), it is reasonable to hypothesize that CyclinB could regulate the occurrence of the cortical MTs. Hence, CyclinB could play a role in bcd mRNA gradient formation.

1.8.3 Egg activation

Translational recruitment of maternal mRNAs is an essential process in most biological systems. Activation of mature oocytes could therefore serve as a switch for subsequent translational activities after Drosophila meiotic arrest. Two genes, cortex and grauzone, which were identified in a screen for female-sterile mutations, play roles in translation and poly(A) tail length determination of bcd mRNA (Harms et al., 2000; Page and Orr-Weaver, 1996; Schupbach and Wieschaus, 1989). Embryos laid by either cortex or grauzone mutant mothers contain a shorter poly(A) tail of bcd mRNA (~ 80 nucleotides versus ~140 nucleotides in wild type embryos), and the post-translational activity of bcd mRNA is disturbed (Lieberfarb et al., 1996). (Horner et al., 2006) reported that bcd mRNA translation is repressed in eggs laid by sarah mutant mothers (sarah encodes the Drosophila calcipressin involved in egg activation) by interrupting the polyadenylation machinery, as well.

1.8.4 Drug treatment of actin and MTs

Actin participates in protein-protein interactions more than any other recorded protein. If Drosophila oocytes were bathed in the actin-depolymerizing drug cytochalasin D, bcd mRNA localization at the anterior tip was compromised and stable actin-dependent anchoring of the mRNA was no longer possible (Weil et al., 2006; Weil et al., 2008). There are various actin-targeting drugs used in basic research (Allingham et al., 2006; Spector et al., 1999). Of note are the latrunculins, an actin-destabilizing drug similar to cytochalasin D used in the past to disrupt filamentous actin (F-actin) in early Drosophila embryos. Phalloidin, a F-actin-
stabilizing drug is used predominantly as a tool to visualize F-actin in combination with fluorescent phalloidin-derivatives.

Small commonly-used molecular MT-binding drugs (Florian and Mitchison, 2016), summarized in Table 2, have made significant impacts in both in medicine and basic research (Dostal and Libusova, 2014), (Fig. 14). The use of drugs that assemble or destroy MT arrays, such as Taxol, Vinblastine, Colcemid and Colchicine has been important in studying the mechanism of the MT network for transportation of bed mRNA (Fahmy et al., 2014; Weil et al., 2010). However, the waxy layer that encompasses the vitelline membrane of the Drosophila eggshell poses a challenge as to the use of drugs, by reducing the permeability. Fortunately, several methods for permeabilizing the embryos still in their vitelline membrane were described (Rand et al., 2010; Schulman et al., 2013).

<p>| Table 2 Summary of drugs acting on MTs |
|----------------------------------------|---------------------------------|-------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Drug</th>
<th>Binding site</th>
<th>Textbook mechanism of action</th>
<th>Effects on MT array</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colchicine</td>
<td>Colchicine</td>
<td>Inhibits neutrophil migration</td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Colcemid</td>
<td>Colchicine</td>
<td></td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Combretastatin A4</td>
<td>Colchicine</td>
<td>Antiangiogenic</td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Colchicine(?)</td>
<td></td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Benomyl</td>
<td></td>
<td></td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>T138067</td>
<td>Binds covalently to Cys-239 of β-Tubulin</td>
<td></td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>Vinca</td>
<td>Antimitotic</td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Vinca</td>
<td>Antimitotic</td>
<td>Inhibits MTs polymerization</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Taxane</td>
<td>Antimitotic</td>
<td>Polymerizes MTs</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>Taxane</td>
<td>Antimitotic</td>
<td>Polymerizes MTs</td>
</tr>
<tr>
<td>Eribulin</td>
<td>Vinca</td>
<td>Antimitotic</td>
<td>Inhibits MTs polymerization</td>
</tr>
</tbody>
</table>
Fig. 14 Binding sites for main MT drugs
Binding site of Colchicine is shown in the red zone. The blue zone is the binding site of Vinca, and the green zone is responsible for binding of Taxane, respectively. Image from (Dostal and Libusova, 2014)
1.8.5 Hypoxia in *Drosophila* embryos

The reaction to low-O\textsubscript{2} environment is diverse among animals. Some species in the invertebrate kingdom, as exemplified by *Drosophila*, are capable of being exposed to hypoxic conditions for prolonged periods without apparent tissue injury. During the hypoxic state, *Drosophila* embryos stop development and enter into an arrested or a “sleeping” phase that can be reversed upon being exposed in oxygen again (Haddad et al., 1997). However, (Heinrich et al., 2011) reported that the growth rate and the body size are affected, generating smaller-sized thoraces and wings by multiple mechanisms at various developmental stages.

1.9 The GAL4-UAS system for inducible gene expression in *Drosophila* research

Manipulation of gene expression is an important tool for studying the function of a gene. A binary expression system is a popular and efficient strategy to up- or down-regulate a target gene (Riabinina and Potter, 2016; Viktorinova and Wimmer, 2007). In *Drosophila*, the GAL4-UAS system has been an extraordinarily efficient tool to dissect gene function in subsets of cells within a given tissue (Brand and Perrimon, 1993; Fischer et al., 1988). The working principle of the system is: an enhancer-driven GAL4 gene that encodes a yeast transcriptional activator will specifically bind to a region where the UAS (upstream activating sequence) is located. The cDNA sequence of a targeted gene \(X\) is placed downstream of \(UAS\) (Fig. 16) (St Johnston, 2002). In *Drosophila*, the binary system is achieved by crossing one strain carrying the GAL4 driver to another strain carrying the \(UAS\)-gene \(X\) construct (Fig. 15). Combined with extensive RNA interference (RNAi) stock collections and classical genetic mutations, the GAL4-UAS system has proven itself as a very good tool to directly assess the effects of altering the activity of a single gene in most developing tissues.
Fig. 15 The GAL4-UAS system.
The yeast transcriptional activator GAL4 can be used to regulate gene expression in Drosophila by inserting the upstream activating sequence (UAS) to which it binds next to a gene of interest (gene X). The GAL4 gene has been inserted at random positions in the Drosophila genome to generate ‘enhancer-trap’ lines that express GAL4 under the control of nearby genomic enhancers. Therefore, the expression of gene X can be driven in any of these patterns by crossing the appropriate GAL4 enhancer-trap line to flies that carry the UAS–gene X transgene. Image from (St Johnston, 2002)
2 PRESENT INVESTIGATIONS

2.1 Paper I: αTubulin 67C and Ncd are essential for establishing a cortical microtubular network and formation of the bicoid mRNA gradient in Drosophila

Aim and results

The Bcd protein serves as a paradigm for morphogen gradient formation in textbooks and has been studied during more than 3 decades. Two competing models of how the gradient arises are available: the SDD model (Synthesis, Diffusion and Degradation) and the ARTS model (Active RNA Transport and Synthesis). The SDD model proposes that the Bcd protein is synthesized from an anteriorly-localized bcd mRNA, and subsequently diffuses throughout the embryos to establish the gradient, combined with uniform degradation (Driever and Nusslein-Volhard, 1988). In contrast, the ARTS model proposes that the bcd mRNA is actively transported along the cortex to the posterior to form an mRNA gradient that servers as a template for Bcd synthesis (Spirov et al., 2009). The existence of a bcd mRNA gradient was first identified by (Frigerio et al., 1986).

The SDD model recently encountered a big challenge (Gregor et al., 2007), so the question of how the bcd mRNA gradient up to 40% EL (Fig. 1A, B) forms was answered in this paper.

Using a monoclonal antibody YL1,2 made against freshly-assembled Tubulin, a cortical and omnidirectional MT network was detected that appeared only in the anterior half of the embryos during metaphase and early anaphase of nc 1-6 embryos (Fig.1C, F). The network was not built up in unfertilized eggs (Fig. 1D, E). Moreover, as embryonic development proceeded, the cortical MTs became more and more numerous. At nc 6, the MT threads were detected to extend 20 µm from the cortex (Fig. 1H, H’). At nc 11, the threads became denser below the nuclear monolayer (Fig. 1J, K). At nc 13, vigorous MT activity was found showing long extensions up to 30 µm into the interior (Fig. 1L). At nc 14, threads extended further into the yolk with a length up to 50 µm (Fig. 1M). Interestingly, the majority of those long threads were also detected using an antibody against αTubulin 67C (Fig. 1O), a maternal Tubulin known to contribute to long MTs.
Given the fact that αTubulin 84B and αTubulin 84D preferred to be associated with MTs that embrace the nuclei (Fig. 1P), it was proposed that the composition of αTubulins in the different cellular sub-compartments varies in space and time. Furthermore, a study between the relationship of actin and the MT network revealed that the two networks were independent of each other (Fig. 2).

Furthermore, among all 4 αTubulin genes, the expression profile of αTub67C was remarkably similar to that of bcd mRNA (Attrill et al., 2016). Thus, we explored the role of αTub67C for the cortical MT network. We stained eggs of kavarnull/Df hemizygous mothers (a complete knock-down) and found many short MTs without orientation (Fig. 4A, B). Next, we looked at the role of αTub67C for bcd mRNA transport and showed that in αTub67C mutants, instead of being transported along the cortex, bcd mRNA moved in the interior of the embryos (Fig. 4C). In search for specific motor proteins associated with αTub67C, we found that nonclaret disjunctional (ncd) encoding an unconventional minus-end Kinesin interacted genetically with αTub67C. Analyses from both strong and weak ncd alleles showed similar bcd mRNA movement as seen in kavarnull mutations (Fig. 4F). To corroborate an involvement of ncd in the transport of bcd mRNA together with αTub67C, we stained embryos with antibodies specific for each protein alone. A clear colocalization between Ncd and αTub67C was demonstrated in the periplasm and perinuclear areas of nc 14 embryos (Fig. 4P, Q), suggesting that indeed these two proteins interact with each other.

2.2 Paper II: Cortical movement of Bicoid in early Drosophila embryos is actin- and microtubule-dependent and disagrees with the SDD diffusion model

Aim and results

Following the work in 2014 (Fahmy et al., 2014), the ARTS model strongly challenged the SDD model. In the ARTS model, the bcd mRNA is transported along the cortical MT network to generate the mRNA gradient, which then serves as template for translation of the Bcd protein. To further provide evidence in support for the ARTS model, we developed a sensitive assay to monitor the movement of Bcd during early nuclear cycles.

The SDD model predicts diffusion of the protein from the anterior tip throughout the embryo, including the cortex as well as the interior yolk. To achieve a high sensitivity, we used a sensitive antibody against Bcd, combined with a strain that expresses 3 times more Bcd protein than in wild-type embryos, bcd5+8. As
expected, the Bcd protein gradient formed gradually from stage of nc 6 to stage of nc 9 (Fig. 1A to D). However, we observed that the Bcd gradient always remained along the cortex and no traces of Bcd were found in the interior of the embryo. To further investigate the movement and the path of Bcd along the cortex, we exposed embryos to hypoxic conditions, which would inactivate the mRNA transportation system without affecting the viability of embryos. We chose nc 6 as a reference time-point to evaluate Bcd migration from the position where hypoxic conditions were applied. Embryos collected from different time periods of hypoxic treatment revealed that the Bcd movement was still at the cortex, but its movement was remarkably sparse (Fig. 1E to I). In contrast, the bcd mRNA remained tightly located at the tip with virtually no movement (Fig. 1K, L). We then analyzed the developmental consequences of the extra movement of Bcd under hypoxia. The majority of embryos exhibited an enlargement of anterior segments with squeezed posterior segments (Fig. 1M), in contrast to a smaller portion of embryos missing several segments (Fig. 1N). Interestingly, the formation of pole cells was suppressed (Fig. 1Q). To investigate whether Bcd movement is MT-dependent or not, we combined our water-based hypoxic treatment with drug application. Embryos exposed to hypoxia and the MT-destabilizing drug mixture colchicine/colcemid (CC) displayed extensive interior movement during the developmental stages (Fig. 3A-C). Embryos treated with the MT-stabilizing drug taxol maintained the Bcd movement along the cortex (Fig. 3E, F). Surprisingly, when embryos were treated with the drug vinblastine, Bcd was distributed throughout the embryos (Fig. 3K). This data indicates that indeed the SDD model could be simulated experimentally, but only if the yolk was modified. To rule out that the Bcd protein movement resulted from movement of the mRNA, we stained all the different treatments for the presence of bcd mRNA (Fig. 3D, H, L). In all cases, we detected little movement, i.e. a similar distribution with that of hypoxia-only treated embryos (Fig. 1K).

To investigate whether actin participates in the movement of Bcd, we subjected bcd\(^{5+8}\) embryos to drugs that disturb the formation of actin filaments. Embryos exposed to the actin-destabilizing drug latrunculin B (Fig. 4A-C) revealed sparse cortical movement with no interior Bcd movement. Embryos treated with the actin-stabilizing drug phalloidin (Fig. 4E-G) behaved like bcd\(^{5+8}\) embryos with a minor fraction of protein moving to the interior. As expected, the bcd mRNA from treated embryos with either drug stayed at the tip with little or no movement (Fig. 4D, H).

In addition to investigating the roles of cytoskeleton actin and MTs, we analyzed the behaviour of Bcd and Staufen protein (as a read-out system of bcd mRNA) in unfertilized embryos during short time intervals to monitor their movements. Our results indicated that the Bcd protein followed an interior path, which paralleled that of the pattern of bcd mRNA (Fig. 5). Our data suggested that an internal MT
network exists in unfertilized embryos that served as a transportation path for the \textit{bcd} mRNA, while the cortical MT network and cortical mRNA transport would be established only after fertilization.

2.3 Paper III: Genetic analysis of factors influencing \textit{bicoid} gradient formation in \textit{Drosophila}

**Aim and results**

Substantial evidence was shown that \textit{bcd} mRNA gradient formation underlies the Bcd protein formation during \textit{Drosophila} embryogenesis. The transport mechanism of \textit{bcd} mRNA in embryos is MT-based and one motor protein was identified (Fahmy et al., 2014). However, knowledge on other factors that influence \textit{bcd} mRNA gradient formation is sparse. Given our previous and current studies, we investigated four groups of factors, \textit{trans}-Golgi proteins, poly(A) polymerases, Cyclins and egg activation genes, in order to pave a fundamental path to the mechanisms of \textit{bcd} mRNA gradient formation.

We stained MTs with a MT-plus end marker, Chromosome bows (Chb), formerly called Mast/Orbit/CLASP. We could show that Chb was associated with cortical MTs in \textit{Drosophila} embryos (Fig. 1). Since Chb was shown to serve as an aMTOC, in concert with \textit{trans}-Golgi proteins in vertebrates, we investigated whether some of the proteins from the \textit{trans}-Golgi compartment were involved in cortical MT-network establishment in \textit{Drosophila}, as well. Using genetic approaches to over-express and down-regulate candidate proteins, we analyzed the effects by monitoring the patterns of \textit{bcd}-downstream genes such as the gap gene \textit{empty spiracles (ems)} and the pair-rule gene \textit{even-skipped (eve)}. We analyzed 4 \textit{trans}-Golgi genes, \textit{dGCC88}, \textit{dGolgin97}, \textit{dGCC185} and \textit{dGolgin245} and could demonstrate that 3 of them affect the Bcd gradient. Summarizing the cuticle patterns in the 4 \textit{trans}-Golgins (Fig. 2), we could observe that all four mutations revealed head-defects, likely caused by altered Bcd activity. Furthermore, the embryos from the partial loss-of-function of \textit{dGCC185}^{83847}/Df(3R)08155 showed defective germband retraction (Fig. 2H). A small portion of \textit{dGolgin97} embryos exhibited an interesting phenotype of distinct regional nuclear density at nc 14 (Fig. 4E, insert), which showed two sets of nuclear density along the AP axis.

To investigate the relationship between the length of the of the poly(A) tail of the \textit{bcd} mRNA and gradient establishment, we targeted two poly(A) polymerases, Wispy and PAP2. Due to the lethality of \textit{pap2} overexpression, we were only able to obtain information from \textit{wispy} overexpression (Fig. 5). The poly(A) polymerase
gene *wispy*, presumably acting on the poly(A) tail length of the *bcd* mRNA was found to shift the Eve stripes mainly to the posterior. Due to the effect caused by Wispy acting on various other segmentation genes, the embryos displayed various segmental defects (Fig. 5J, K, L). Furthermore, we performed a study on the cell-cycle gene *CyclinB*. When overexpressed, CyclinB was shown to affect the segmental anlagen primarily in the anterior part (Fig. 6). Finally, to determine the link between egg activation and Bcd gradient formation, we analyzed 3 egg activation genes. Due to high mortality of egg from cortex mothers, we could not execute statistical analyses. Two egg-activating genes (*grauzone* and *sarah*) were shown to affect the Bcd gradient by shifting the majority of the Eve stripes to the posterior.

In summary: we have provided evidence of the existence of factors with diverse cellular functions that regulate Bcd gradient formation. Our data reveals a larger complexity of the mechanisms for Bcd gradient formation than initially anticipated.

### 2.4 Paper IV: Segmentation gene expression patterns in *Bactrocera dorsalis* and related insects: regulation and shape of the blastoderm and larval cuticle

**Aim and results**

Previous studies revealed that *bcd* diverged recently from the *Hox3* gene in the course of insect evolution, instead of being conserved as the other *Hox* class genes in animals (Stauber et al., 1999; Stauber et al., 2002). It is well known that *bcd* is the most important anterior determinant for the generation of the AP polarity in *Drosophila* embryos. However, *bcd* is not detected in many other flies, and plays its role only in higher dipterans (Bonneton, 2003; McGregor, 2005). One interesting avenue was therefore to investigate is how the AP patterns look like in other flies where *bcd* was absent.

In this study, we explored the expression patterns of the *bcd* target genes in the oriental fruit fly *Bactrocera dorsalis*. Phylogenetically, *B. dorsalis* is a member of the family *Tephritidae* that is located immediately adjacent of that of *Drosophilidae* (Geib et al., 2014). The (at that time) unpublished data showed that the *bcd* gene was lacking in the *B. dorsalis* genome. Since the embryology of *B. dorsalis* was not described in detail, we first undertook a comparison of its embryology to that of *Drosophila*. This showed that the embryogenesis of *B. dorsalis* was very similar to that of *Drosophila*, except that the overall shape of the
embryo was quite different (Fig. 1 and 7B). Next, we examined the expression patterns of selected *bcd*-downstream target genes in *Drosophila* that were also expressed in *B. dorsalis*, including the gap genes *hb*, *otd* and *Kr*, the pair-rule gene *eve*, and the segment polarity gene *engrailed* (*en*). Interestingly, the onset of expression of all segmentation genes was detected one nc later, compared to their *Drosophila* counterparts. Moreover, while the transcription of *otd* continued in *Drosophila*, *otd* transcripts in *B. dorsalis* ceased after the extended germ band stage. Among those expression patterns of the genes, the way that the *eve* stripes were aligned along the AP axis was particularly conspicuous. It was perpendicular to the AP axis (Fig. 5), suggesting a distinct control. Furthermore, 3 extra stripes of *en* were observed in *B. dorsalis*, which were not found in *Drosophila* (Fig. 6). Finally, a comparative expression analysis revealed a distinct interpretation of the gap gene (*Kr*) signal at the pair-rule level (*eve*) between *B. dorsalis*, *Drosophila* and a third fly, the blow fly *Lucilia sericata* (Fig. 7).
3 ACKNOWLEDGEMENTS

There is an ancient Chinese saying: as one fence needs three stakes, a good guy needs three fellows. Here, I would like to express my sincerest gratitude to those who have supported, encouraged me to a successful PhD.

Stefan Baumgartner: my supervisor. ‘Please do not call me Dear Professor Baumgartner, just Stefan.’ I will always fondly remember you and Ulrike picking me up at the airport. I recalled being so exhausted but immediately felt comfortable in the way you spoke that it put me such at ease while introducing me to an unfamiliar town. For one who is used to the hierarchial system, instinct told me that my PhD life in your group would be more enjoyable, which it has been proved to be during my 4 years. With your patience, tolerance, and kindness, I adapted myself well and walked the track of studying this discipline more smoothly. Despite my uninformed questions at times, you have always had time to explain them as detailed as possible, providing corresponding solutions. Regardless of my errors, you have always smiled and said: ‘learn it next time.’ Undeterred by unexpected results, you have always taken the most optimistic viewpoint. You keep the working environment both professional and friendly, not only in the lab, but your cordial attitude extends to neighboring labs and even the entire floor. Under such conditions, have I been more relaxed and more efficient. Thank you for offering me such a constructive working environment to satisfy my curiosity in developmental biology as a discipline. More importantly, thank you for being a good life mentor, I have learned a lot from you, both academically and socially. Whatever lies in store for the future, let this not be goodbye.

Sol Da Rocha Baez: techniques tutor. ‘I do not trust people with a 24-hour smile.’ Even though it was not a judgment about me, I was worried if I could ‘survive’ under your military-like working style. Happily, I did. With your help and guidance, I have greatly improved my experimental skills, and have become more rigorous. By the way, I kept that Swedish pizza menu translation that you wrote the first time for our routine Friday weekly lunches. After so many years, it has greatly assisted me when ordering food.

Udo Häcker (co-supervisor) and Martina Schneider: my Drosophila neighbors. Without your company, I am pretty sure that our flies would feel too lonely in the BMC. Thank you for making us happy. Thank you for sharing your wisdom to us. Because of your presence, I have learned a lot about the German logic and
precision. Because of your presence, I have also been familiarized with the ‘not-funny-at-all’ German humor.

**Edgar Pera:** the Frog. ‘You guys are so anti-social. You have to put Fika on the top of your daily schedule. That is the priority on Wednesday.’ Ha-ha, too much pressure from your friendly fly-friends, but so much fun for us to just tease your group that way.

**Maria Climent:** my dear JieJie. The first time I met you, you simply gave me a big and sweet smile. From that moment, I knew if I had any problems, you were the person I could go to for help. You brought the Spanish sunshine to my first dark winter here. It was so grateful that you invited me to spend Christmas with your and also my Spanish family together. I am so happy to have a sister like you.

**Linda and Thomas Blom, Sara Andersson:** ‘Linda, Linda, listen-listen to me!’ Thank you for your generously revising my thesis and transcripts and more transcripts and then thesis again! Thank you for sharing your fruit basket with such a ‘poor student’. I hope soon Linda will manage her Swedish, otherwise it would definitely be the fault of Thomas and Sara! Ha-ha!

Time flies. Within those 4-years, so many people have come and gone. **Khalid Fahmy** (your delicious baklava and broad knowledge of insects), **Worramin** (my Thai sister and your awesome Oriental Fruit Fly), **Lukas** (‘guinea pig’ of my food experiments), **Inge** (finally my ‘labor’), **Nadège** (wondering what could knock you down), **Franziska** (the first German with blue eyes) and **Cibely** (Brazil sunshine and nice host in Barcelona).

Some became friends, some were just passersby. But, whatever their roles are, they played a very important part through a certain stages of my life. I am so grateful that I have met all of you guys. I am a life-experience collector. Those precious memories will be kept close at reach and close to my heart.
4 REFERENCES


Introduction

The Bicoid (Bcd) protein gradient in *Drosophila* serves as a paradigm for gradient formation in textbooks. To explain the generation of the gradient, the ARTS model, which is based on the observation of a local mRNA gradient, proposes that the bcd mRNA, localized at the anterior pole at fertilization, migrates along microtubules (MTs) at the cortex to the posterior to form a steady mRNA gradient which is translated to form a protein gradient. To fulfill the criteria of the ARTS model, an early cortical MT network is thus a prerequisite. We report hitherto undiscovered MT activities in the early embryo important for cortical transport of the bcd mRNA, which make predictions of the diffusion coefficient problematic. It is thus important to note here that, although these above experimental and theoretical data permit to explain the SDD model, it would be premature to imply that the SDD model predicts long-range diffusion of Bcd. Arguably, the diffusion properties of proteins in the dense yolk are different from those of the cytoplasm surrounding the cortical nuclei at 10–14 nuclear cycles ([8]). Unfortunately, as a major drawback, all the above analyses comprised measurements of diffusion during late nuclear cycles (nc) 10–14 and at the peripheral cytoplasm of the embryo. However, we should bear in mind that the time window from fertilization up to nc 10 is the important time interval where the SDD model predicts long-range diffusion of Bcd. Moreover, the diffusion properties of proteins in the dense yolk are different from those of the cytoplasm surrounding the cortical nuclei at 10–14, which may produce predictions of the diffusion coefficient problematic. It is thus important to note here that, although these above experimental and theoretical data permit to explain the SDD model, it would be premature to imply a priori that the SDD model is the correct one. Furthermore, the model still lacks a direct proof of the existence of long-range Bcd diffusion, e.g., by tracking single Bcd molecules during the early nuclear cycles. Equally important to note: the ARTS model does not argue against a high diffusion coefficient of Bcd, it is only largely irrelevant for the occurrence of the local mRNA gradient.

Abstract

The Bicoid (Bcd) protein gradient in *Drosophila* serves as a paradigm for gradient formation in textbooks. To explain the generation of the gradient, the ARTS model, which is based on the observation of a local mRNA gradient, proposes that the bcd mRNA, localized at the anterior pole at fertilization, migrates along microtubules (MTs) at the cortex to the posterior to form a steady mRNA gradient which is translated to form a protein gradient. To fulfill the criteria of the ARTS model, an early cortical MT network is thus a prerequisite. We report hitherto undiscovered MT activities in the early embryo important for cortical transport of the bcd mRNA, which make predictions of the diffusion coefficient problematic. It is thus important to note here that, although these above experimental and theoretical data permit to explain the SDD model, it would be premature to imply a priori that the SDD model is the correct one. Furthermore, the model still lacks a direct proof of the existence of long-range Bcd diffusion, e.g., by tracking single Bcd molecules during the early nuclear cycles. Equally important to note: the ARTS model does not argue against a high diffusion coefficient of Bcd, it is only largely irrelevant for the occurrence of the local mRNA gradient.
The existence of a lcf mRNA gradient, which is the hallmark of the ARK model, was first described in 1988 [7]. In 2001, the SSD model was challenged by a detailed analysis of the lcf mRNA distribution during early Drosophila embryogenesis [8], which confirmed previous data [7]. On the other hand, the ARK model, which is based on this demonstration, raises the question of how the lcf mRNA gradient forms from a lcf mRNA source that at fertilization is centrally localized to the anterior pole of the embryo.

Plenty of information is immediately available on the transport of the lcf mRNA during embryogenesis, using in vivo imaging of the movement of the lcf mRNA [9,10]. These data showed that the changing of the lcf mRNA by the microtubule transport processes as it is extruded from the embryo, that is associated with the germ plasm, and that at fertilization the lcf mRNA is delivered to the anterior pole [10]. However, it is important to note that at the end of oogenesis, the lcf mRNA is confined completely to the anterior pole, denoted by the aster cytoplasm. Recent measurements showed that Drosophila females produce around 7 x 10^10 lcf mRNA molecules which are dropped into the egg as maternal supply [11].

As far as the MIT network in the oocyte is concerned, several conflicting models were published, which have been organized and how they transport maternal factors to the anterior or posterior pole of the oocyte [12-17]. More recently, however, using in vivo imaging, [12] et al. resolved some issues of the conflicting models and reported highly dynamic motion that are organized with a biased random polarity that increased towards the posterior [16]. However, it is important to note that at the end of oogenesis, all MTs are completely degrading again [17-19], implying that the fertilized embryos need to start to build up the MTs from the beginning.

The ARK model postulated a mechanism based on MTs that transport the lcf mRNA [1]. Yet, for the convincing evidence for such a postulated network of MTs has been lacking. Patteron et al. resolved some issues of the conflicting models and reported highly dynamic motion that are organized with a biased random polarity that increased towards the posterior [16]. However, it is important to note that at the end of oogenesis, all MTs are completely degrading again [17-19], implying that the fertilized embryos need to start to build up the MTs from the beginning. Therefore, we present a method for that would allow to prepare the anterior pole of the oocyte MTs as early as possible. This fixation technique, along with monoclonal antibodies to tubulin, is a method to visualize the entire oocyte, including the anterior pole. In addition, this network was confirmed exclusively to the anterior half of the embryo and appeared only during mitosis and anaphase of the 1-8 embryo (Fig. 1C, insert), i.e., during later stages of the embryo [17]. Moreover, the networks were found to be present between 30 mm and 35 mm of the nuclear cycle (Fig. 3E, F). Attempts to live image the network using a tubulin-GFP-GFP embryos failed, due to the weakness of the signal and the dynamics of the MTs (data not shown). The network was not detected in unfertilized embryos (Fig. 1D, E), but became apparent from Z: onwards, exemplified by a see: 1 (Fig. 1C) or a see: 4 embryo (Fig. 1G). From see: 6 on, it was present throughout all nuclear phases, e.g., during interphase (Fig. 1H, I), while still being absent in the posterior half (Fig. 1I). At see: 7, when cortical migration commences, the posterior half showed cortical MT activity also (data not shown). At see: 11, tyrosinated tubulin is detected in a dense network below the nuclear layer originating from long axonal MTs [20,21] (Fig. 1K). Notably, these axonal MTs showed strengthened lcf mRNA binding activity [20]. At see: 13, strong MT activity was detected showing long extensions up to 30 mm into the yolk (Fig. 1L, insert) and even longer ones in see: 14 embryos (Fig. 1M, N, up to 50 mm). These threads resemble those on the drawings by [19]. The majority of these long MTs could also be stained using antibodies against nTubulin [21] (Fig. 1C, D). A maternal Tubulin known to contribute to long MTs and which is responsible in the cytoskeleton of cells in early embryos, involving nTubulin and a maternally expressed nTubulin is a key factor in the stabilization of the mitotic spindle. Therefore, we also showed a striking reorganization in Tubulin; it existed with nTubulin, which drives both the yolk and the mitotic spindle (Fig. 1P, Q), and a Tubulin, which is associated with the axonal MT (Fig. 1P, green channel), compared in nTubulin, which also accumulates in the mitotic spindle (Fig. 1G, green channel). Hence, the composition of Tubulin is different in the different sub-compartments of the yolk, which is illustrated by the observation of long MTs in earlier stages of the embryo.
that the long MTs in Fig S-J-M were preserved owing to the
improper fixation method.

Independence of the MT network from actin

To determine whether or not a link exists between the cortical
MT network and actin, we examined their relative distribution
patterns in early embryos. In the anterior tip of a 2-cell embryo, the
actin formed a dense layer (Fig S-K-A) which was not in contact with
the MT network (Fig S-K-C). At later stages (Fig S-L), the situation
remained unchanged (Fig S-L-A). A 3-D visualization technique of
the two cortical marks in Fig S-A and B showed us to visualize
the relationship of the MT threads to actin more explicitly
(Fig S-M, N, Video S-2 in SI). Exclusively, at the locations of the
tail area, the MT network was associated with the actin sheet,
either outstretched immediately next to it or the inner cortex.

Takai induces antarsis

Previous analyses of MT activity reported a cortical network of
short MT (22,33,35), but many of these investigations used the
MT-disrupting drug Taxol. We therefore repeated these experi-
ments with Takai and noted that the MT threads could even be
detected in unfertilized eggs (Fig S-A, B). Furthermore, the
threads were found unevenly distributed in the posterior half.
Fertilized embryos showed the same distribution, as shown in a 5-
cell embryo, although the density of threads was somewhat
lowered, while no difference in length was detected (Fig S-C, D).

Staining with Rhodamine Phalloidin+Calcein revealed the
distribution of MTOF threads (Fig S-C, D, F). Our data from Takai
treatment suggest that the Takai induces a movement of MTs
are present throughout the embryo, but under normal conditions
they polymerize only in the anterior half.

Xutub676C and ndc are critically important for cortical
MT formation and bid mRNA transport

Of the Takai genes (34), that would be critical for cortical
mRNA transport, we considered Xutub676C a good candidate since
it showed maternal expression and an expression profile similar to
that of kcd mRNA (54,35). The Xutub676C locus is represented by
a tub676C mutant allele (28,36) and by the independently
isolated dominant female sterile kcd mutant (31,37). We
created oogenic kcd+/+; Xutub676C homozygous mothers (a
complete loss-of-function LOF) matings (38) for the presence of the
cortical network and found a network of short MTs without directional
(Fig S-A, B, C). Our results suggest that the maternal expression of
Xutub676C is required for the formation of long MTs (21,37). In
contrast, the Xutub676C mutant allele, tub676C, did not localize to
the anterior pole, resulting instead at the edge (Fig S-D). In a late stage
14-somite, the aberrant localization was even more pronounced showing a
darker anterior distribution (Fig S-E), demonstrating a vital role in
ndc in anterior localization of the bid mRNA during
organogenesis, in contrast to the wild alleles, as above. In Xutub676C
kcd+/+; Xutub676C mutants, the mRNA showed a less gradual gradient from
the site of fertilization on, similar to the profile in bid eggs (Fig S-F,
G, H), data not shown. However, when the Xutub676C embryo were
incubated in the presence of cortically cultured embryos, as
above, the mRNA showed a weak and localized distribution in the
anterior pole as compared to the bid mRNA (Fig S-I, J). This
suggests that bid expression is dependent on the maternal
expression of Xutub676C.
Figure 1. The accumulation of mRNAs and an anterior cortical microtubule network. (A) A single confocal section of a nuclear cycle (nc) 14+ 4 min. Different embryos showing a typical accumulation of mRNAs (green) extending up to 30% of the egg length (SE scale bar below embryo). (B) Representative confocal section of an egg showing the mRNAs (green) and the integrity of the cortical actin network (red). The asterisk denotes the cortical actin. (C) Higher magnification of the dotted region of an embryo in (A), numbers of (N) denote the nuclei. (D) Confocal analysis at the surface of an nc 14+4 embryo at enlarged (30%) egg length showing the accumulation of actins (red) and microtubules (blue), a filamentous network of cortical actins, and two pole bodies (PB). Two adjacent confocal sections 2.4 μm apart and maximal intensity projection were used. White areas denote magnifications of corresponding anterior (at) regions and posterior (post) regions used in (E–G, J–K). (F) Magnification of anterior (at) and posterior (post) portions on the surface of an unfertilized embryo. No network is visible. (G) and (K) Magnification of anterior (at) and posterior (post) portions on the surface of an embryo at nc 4. The network is visible at the anterior half, but is absent in the posterior half. (H), (J), and (L) Sagittal confocal sections of the anterior (A) and the posterior (P) half of an embryo at nc 8. (M) Magnification of the area indicated in (H). The MT threads are exclusively at the anterior cortex (arrow). In the interior, asters of interphase nuclei are seen. (O) Confocal section just below the nuclear layer of a nc 11 embryo and (O) mid

that the long MTs in Fig. 3-M were preserved owing to the
impressed fixation method.

Independence of the MT network from actin.

To determine whether or not a link exists between the cortical
MT network and actin, we examined their relative distribution
patterns in early embryos. In the anterior tip of a 2-7 embryo, the
actin formed a dense layer (Fig. 2A) which was not in contact with
the MT network (Fig. 2B, C). At later stages (3-6), the situation
remained unchanged (Fig. 3D-F). A 3-D visualization technique
of the two cortical markers in Fig. 2A and B allowed us to visualize
the relationship of the MT threads to actin more explicitly
(Fig. 2G, H). Video 2 (3 GB). Exclusively, at no locations of
the indicated area, the MT network was associated with the actin sheet,
or rather raised transiently next to it in the inner cortex.

Takol induces artefacts.

Previous analyses of MT activity revealed a cortical network of
short MTs [23,33,35], but many of these investigations were
the MT-stabilizing drug Taxol. We therefore repeated these experi-
ments with Takol and noted that the MT threads could even be
detected in unfertilized embryos (Fig. 3A, B). Furthermore, the
threads were found uniformly distributed in the posterior half
of the embryo. The actin showed the same distribution, as it does in a
nc 5 embryo, although the density of threads was somewhat
increased. While no difference in length was detected (Fig. 3G, H),
the number of cortical MTs with Confocal microscopy, drug-induced
dense MT threads were not detected in Takol-induced MT threads, was applied, we found that the drug
truncated MTs were not associated with any specific localization of the cell cortex.

In our data from Takol treatment suggest that the Takol
modified the surface of the actin cortex, but not normal conditions they polymerize only in the anterior half.

nTubulin and actin are critically important for cortical
MT formation and bud mRNA transport.

Of the nTubulin gene [34], this would be critical for cortical
mRNA transport, we considered that nTubulin were a good candidate since it
described an expression pattern similar to that of bud mRNA [41,53]. The nTubulin locus is expressed by the Ntub1-10 nonsense allele [28,39] and by the independently
isolated dominant female sterile kissin mutants [11,23].

We crossed nTubulin-kissin homozygous mothers (a complete loss-of-function LOF) mating for the presence of the cortical network and found a network of short MTs without directionality
(Fig. 4A, B). Notably, the observation that nTubulin (nTub 1-10) was required for the formation of long MTs [11,37].

We used the nTubulin allele, nTub1-10, as an example, rather than the mRNA formed a long streak in the interior of the embryo, in parallel to the AP axis (Fig. 4C). Instead, we observed the behaviour of the mRNA in oocytes of homozygous nTubulin females, the weakest available allele. In a stage 10 nTubulin-10 oocyte, the mRNA did not localize to the posterior pole, existing instead at the edge (Fig. 4D). In a late stage 14 oocyte, the aberrant lateral
localization was even more pronounced showing a smaller anterior distribution (Fig. 4E), creating with a flash for a nTubulin-10 allele.

To further correlate an involvement of nTubulin in the transport of bud mRNA, we over-expressed nTubulin during oogenesis using the maternal driver VD5. At a stage 10 oocyte shown lateral accumula-
tion of the mRNA (Fig. 4M, similar to nTubulin-10 oocytes.
Figure 2. Independence of the early MT network from the actin sheet. (A–C) mid-sagittal confocal sections of the anterior tip of a nc 2 embryos stained with Phalloidin (B) to reveal the actin structure, with mAb YL1/2 against phosphorylated Tubulin (B) and merge in (C). (D–F) mid-sagittal section of a ventral region about 50 μm away from the anterior tip of a nc 6 embryos, stained with Phalloidin (D), mAb YL1/2 (E) and merge in (F). (G–I) 3D reconstruction of the confocal stack of the embryos of (A–C), view is from the middle (M) to the more lateral (L) part of the embryo. For images of 3D view, see Video S2. (H) 3D reconstruction of the confocal stack of the embryos of (D–F), view is identical as in G. For films of 3D view, see Video S3. The red background on the inner “nost” of the embryos in (H) and (I) is excess of free tubulin which could not be removed during background subtraction of the 3D program. Stages of embryos are denoted in yellow and follow the nomenclature of [31].

doi:10.1371/journal.pone.0120352.g002

(FIG. 4D) and to a lesser extent also to mAb60 antibody (Fig. 4E). As a result of the aberrant lateral transport during segregation, a nc 6 embryos revealed the mRNA transported more posteriorly (Fig. 4N) compared to a wild-type embryos of the same stage and even more posterior than an identical stage nc10/nc15 embryos (Fig. 4H). In a nc 12 embryos, however, extended transport of the mRNAs in the cortex well beyond the middle of the embryo was observed (Fig. 4O), demonstrating a vital role of nod for the transport of the mRNAs. We also noted a clear bias for accumulation of the ef0 mRNA at the dorsal side in embryos, exemplified by the position of the myosin motor (Fig. 4G, M, inset), which explains the skew of the ef0 mRNA pool at the time of fertilization (Fig. 1B, insert).

αTubulin67C and Nod show colocalization

To perform our analysis on the proposed molecular interaction of αTubulin67C with nod [40,41], we stained embryos with antibodies specific for each protein alone, along with a third antibody that recognized αTubulin67B and αTubulin67C (Fig. 4P–U). Nod and αTubulin67C showed colocalization in the perinuclear and in perinuclear areas of nc 14 embryos (Fig. 4P, Q). Notably, the perinuclear area showed strong ef0 mRNA accumulation upon head-skip transport (Fig. 4R, S), suggesting that αTubulin67C and Nod play also a role during head-skip transport at nc 14. In contrast, αTubulin67B and D head-skip immediately perinuclei, but were virtually absent from the perinuclei, as evident in Fig. IR. Consequently, only the perinuclear area was positive for all 3 proteins which stained in white (Fig. 4R). A pairwise comparison of Nod/αTubulin67C (Fig. 4T) and Nod/αTubulin67B-D (Fig. 4U),
corroborated this behaviour closely. The above data suggested that NudEL preferentially associated with mRMR/C-positive MT bundles and define these two proteins as essential members of the NudEL mRNA transport machinery.

\textbf{Discussion}

The ARTS model \cite{ARTS} predicted the presence of a cortical MT network in early staged Drosophila embryos where only sparse information of MT distribution was available. To visualize this network, two essential modifications of existing protocols were necessary: i) a presalubrination and fixation protocol permitted to
overcome the limitations of rapid fixation of MT structures in the Droophila egg. Indeed, an antibody with an excellent signal-to-noise ratio that detected tyrosinated Tubulin.

Initially, visibility of the cortical network was limited to a short 2-minute window which let us conclude that it is a highly dynamic and short-lived MT network, consistent with the reported specificity of the mAb against tyrosinated Tubulin [28]. The rapid fixation technique also allowed us to identify activity with MT extensions as long as 30 μm into the yolk at later stages (Fig. 3M, N). The MT threads likely serve to transport foci mRNA molecules from the yolk to the apical side of the nuclei as early as 14 [3]. Another possible function could be transporting lipid droplets, shown to be MT-based [3,4].

Froebly-synthesized Tubulin usually carries a COOH-terminal tyrosine residue as signature which is part of the epitope that is recognized by mAb YL1/2. Mature tubulin shows this terminal tyrosine cleaved off, often referred to as Glo-Tubulin, using an enzyme, Tubulin tyrosine carboxypeptidase (TTPC). If necessary, tyrosination of the de-tyrosinated end using an enzyme, Tubulin tyrosine ligase (TTL) could convert the COOH-end back to its original status (reviewed by [43]). As there is no TTL detected in the Droophila genome, we presume that tyrosinated Tubulin is provided to the egg via the pool of maternally-deposited Tubulin, or de novo synthesis.

Our Tassel experiment in Fig. 3B demonstrated that most previous descriptions of MT activity in the early nc embryos
[22,23,27,31] require a careful re-evaluation. The fact that Treadwell found the MTs were oriented longitudinally in unfertilized eggs (Fig. 3A, B) suggested that the unfertilized egg was competent for MT thread assembly, but lacked an associated progeny trigger, or 'initiator' which is activated only after fertilization.

Attempts to comets the cortical MTs with minus-end motors failed (data not shown), most likely because there is no 'conventional' microtubule-organizing center (MTOC) at the cortex. Nonetheless, the minus-end motors usually detected the MTOCs associated with the internal nuclei without difficulty. Consequently, antibodies against plus-end motors do not stick well following our improved fixation method. We were unable to stain the MTs using Tubulin-GFP antibodies (unpublished), most likely due to the fact that the signal from their free structure is difficult to detect within the autofluorescence of the yolk. The only profile that contains the cortical MT network is Chromosome body (Chb), formerly called MTOC/Ory/CLASPL (Hewson, 1987), a MT-staining protein and a plus-end motor, which decorated the MTs uniformly (data not shown). A recent survey in the literature revealed compelling evidence that MTs can form without functional cytoplasmic [80-86]. Of these, the observation that MTs can be visualized at the trans-Golgi network with the help of vertebrate CLASPL [65] attracted our attention. Indeed, Golgi network structures could be detected at the cortex of early [1-4] embryos [84,85].

As far as the velocity of the full mRNA transport is concerned, the velocity of Siliites of RNPs complexes was measured in the oocyte, resulting in values of 0.96 to 2.15 km/sec [10]. As was discussed in favor of the ARTS model [2], an unidirectional MT network in vivo was predicted to be a prerequisite for the transport system, due to the fact that an AIP-directed MT network would have transported the full mRNA within minutes from the anterior to the posterior pole. As was discussed for the ARTS model [2], a slight direction bias of the MT network, as was observed in oocytes during trans-Golgi mRNA particle transport [27], represents a valid explanation for the posterior transport, but only if the transport is mediated by both plus- and minus-end motors. However, what is most likely, the transport system would persist only during about 30% of the time of an early nuclear cycle.

An important fact is that all MT activities in the oocyte are ablated at the time of fertilization [29,31]. Hence, new MT structures are de novo formed in the fertilized egg. Our data demonstrate the existence of at least two distinct and unreplaced MT networks (the early embryo (i) the cortical network that exerts crucially on the progress of fertilization and the formation of the fertilized egg [10]), the cortical network which rapidly transports the mRNA towards the interior of the embryo, but not requiring function of Drosophila embryo [10]). Hence, the new MT network is located in the middle of the embryo, its detection proves difficult and hence still awaits visualization. Hist for its existence were shown in the initial process of movement of the full mRNA during 3-4, as stated by [86], before the cortical network comes into play which transports the mRNA to the cortex [10]. Interestingly, about 1-5% of all fertilized embryos show absent transport of the mRNA into the interior and towards the dividing nuclei (data not shown), suggesting that this network may correspond to that proposed for apical expansion of oocytes at 4-6 [57].

Fig. 3A-1: It contradicted earlier findings of a basal-to-apical transport of the mRNA during 14-16 and the existence of an apically-located full mRNA [22], before its final degradation takes place. This behavior was agreed in the analysis of [30]. Curiously, embryos G in Fig. 8 of the same report demonstrated strong expression of an apical full mRNA, thus raising the claim of a basal disruption of the mRNA transport at a steady phase. How do we reconcile these opposing results? We realize that the discrepancy between our result demonstrating the presence of an apical mRNA and their result showing absence caused by the fact that many embryos in the Little al. analysis revealed poor preservation of the preimplantation zygotes which appeared largely rubbery (46, best exemplified in embryos E and F of their Fig. 5, respectively). Consequently, little or no fluorescent signal could emerge from the missing tissue. As far as the discrepancy of a long versus a short mRNA gradient is concerned, we assume (i) it is a similar cause owing to the rubber peripheral, and (ii) possibly a general loss of mRNA of the post-implantation embryos. Indeed, the long mRNA gradient is more conspicuous from the apical full mRNA series [2]; Fig. 1A, B. The short observed mRNA gradient led Little et al. to conclude that diffusion of the full protein is still an absolute requirement for fulfillment of the IEG model. On the other hand, our analysis [3]; this report, and that of [24] demonstrated that the mRNA gradient reached a much more posterior extent than is sufficiently large to exclude the necessity to include a long-distance diffusion of the full protein. To reconcile the difference of the posterior extent between the mRNA and the protein gradient, we propose that the mRNA preparations are not equivalent. Furthermore, we envision that the poly(A) tail length of the mRNA out in control of the translation efficiency of full [36] may represent another tool to extend the gradient to the posterior. Thus, we speculate that the length of the poly(A) tail of the full mRNA increases with posterior migration such that the postembryonic period harbors the longest poly(A) tail. As a consequence, only free mRNA molecules would be required to efficiently translate the full protein in more posterior regions, molecules that current in site hybridization protocols are unable to detect. Interestingly, Wigglesworth's poly(A) polyadenylation was recently shown to be part of the full chromosome polyadenylate particle [36]. Possibly, Wigglesworth remains associated with the particles during the posterior migration, while chromatin bound transcription is the poly(A) tail of the full mRNA. Like this, the poly(A) tail length of the full mRNA does not vary with time [10], but may vary with space. To take the question that was raised in the introduction: we demonstrate that the molecular structure of the whole embryo model predicted exist and described two proteins that play critical roles for the transport machinery that forms the aed mRNA gradient. Further, we provide evidence of a long-distance transport of full mRNA particles up to 40% EL [3] culminating in the mRNA gradient which typically displays the gradient protein. Hence, the crucial question of the ARTS model concerning the mechanism by which the full mRNA gradient forms is answered and confirms the earlier postulate of a ME-mediated transport [3].

Materials and Methods

Fly stocks

The nuc(C1) referred to as nuc(R2) as the only known null allele of T3-37, is described in [37] and was used, together with Df(3L)E74A1, to generate embryos without functional Drosophila embryos [1,37]. The other base alleles were described in [25]. The nuc(D1) and nuc(D2) alleles were described in [25], which the nuc(D1) and nuc(D2) alleles were described in [25], respectively, T3-37(C1) null embryos were obtained by crossing nuc(D1) nuc(D2) Embryos were obtained by crossing nuc(D1) nuc(D2) embryos, as described by [41]. The semidifferent nuc(D1) and the over-expression of PEF(D2) nuc(D2) alleles were obtained from
Bloomington. The maternal GAL4-driver line F32 was obtained from the Bloomington lab.

Fixation of embryos to visualize the microtubular network

In order to visualize the cortical network, we modified a recent permeabilization protocol without RNase [25] and fixed embryos for 15 minutes in 37% formaldehyde, followed by gentle devitronization. The network can also be visualized by using a more conventional 2.7% formaldehyde/glutaraldehyde fixation protocol, but with a somewhat poorer preservation of the MTs. Embryos in Fig. 2 were fixed like the other embryos, but were hand-devitronized.

Drug treatments

After dechorionation, embryos were subjected to 10 μg/ml Taxol for 2 minutes, or to a 50 μg/ml Colchicine/10 μg/ml Colcemid mixture for 15 minutes in permeabilization buffer [25], before addition of 37% formaldehyde.

Antibodies

Mouse VlI2, against tyrosinated tubulin (MIBioRx) was used at 1:2000. Anti-TubulinC, rabbit polyclonal antibodies were made against a peptide from amino acids 33-61 of tUL2, affinity-purified and used at 1:400. Anti-TubulinBD goat polyclonal antibodies were made against the last 15 aa of the tyrosine residue of the common COOH-terminus of tUB3B and tUL2BD, respectively, and used as a crude serum at 1:100. DKO was used as a FITC-conjugate (Sigma) at 1:1000 sequentially to mark VlI2, as in Fig. 2B. Polyclonal goat antibodies against Neb (64-72) were purchased from Santa Cruz Biotechnology and used at 1:100. Polyclonal rabbit anti-TubulinC and Mouse antibodies were obtained from V. Zhang and H. Okamoto, respectively, and were used at 1:1000. For actin staining, we hand-devitronized the embryos and used Phalloidin, coupled to Alexa 488 (Invitrogen) at 1:50. For VlI2, we preferentially used Cy3 antibodies coupled to 304 nm blue lasers to obtain an optimal signal-to-noise ratio. All pictures were taken on a Zeiss LSM 710.

Western analysis

0.2-1.5 embryos were extracted on 12% PAGE and probed with Tubulin-specific mAbs and a polyclonal antibody using standard methods, as described in Figure S3.

In situ hybridization

Fluorescent in situ hybridization was used according to [2]. except that RNA probes were used, combined with an Alexa Fluor 488 Signal Amplification Kit (Biotium, A11060). Care was taken to ensure preservation of the periphery by gentle vortexing during the devitronization step and proper fixation during the post-hybridization steps to avoid poor preservation of the periphery as in [56].

Supporting Information

**Figure S1** hcd mRNA gradients in Diptera. (a) A Drosophila embryo in fixation hybridized with a hcd probe and alkaline phosphate to reveal the strict accumulation of the mRNA at the anterior pole. (b) A Drosophila ec14 embryo hybridized with a hcd probe and alkaline phosphate showing an extended gradient. (c) A Locusta larvae ec14 embryo hybridized with a hcd probe and alkaline phosphate showing an extended gradient. (d) A Locusta larvae ec14 embryo hybridized with a hcd probe using fluorescence. Methods and color conversion as in Fig. 1A, B and 3. (TI)

**Figure S2** Mislocalization of the hcd mRNA in tUL2/BD and motor protein mutants. (A) A 1:1 tUL2/BD:tUL2/BD embryo, stained for the hcd mRNA (green) along with DAPI (blue) to reveal a streak of the mRNA. (B) A 1:1 tUL2/BD:tUL2/BD embryo, stained for the hcd mRNA (green) along with DAPI (blue). (C) 5-DQ recombination of the sp of a 1:5 tUL2/BD:tUL2/BD embryo, stained with Neb VlI2 (red) and DAPI (blue) to reveal a dense MT network and actin-like MT bundles without motor (asterisk). The positions of the asterisks are indicated with yellow arrows, one normal motor phenotype is indicated with a white arrow. A movie of this 3D construction is available at Video S4. (E) A 1:5 tUL2/BD:tUL2/BD embryo, stained for the hcd mRNA (green), along with DAPI (blue). (F) A 1:5 tUL2/BD:tUL2/BD embryo, stained for the hcd mRNA (green), along with DAPI (blue). (G) A wild-type stage 10 oocyte, stained for the hcd mRNA (green), along with DAPI (blue). (H) A nematode stage 10 oocyte, stained for the hcd mRNA (green), along with DAPI (blue). The anterior localization is largely normal. (TI)

**Figure S3** Specificity of Tubulin antibodies. Western analysis of 0.5 h embryonic extracts. Figure S3 showed that VlI2 detected two Tubulin bands, in agreement with previous reports [26]. The upper band corresponded to tUB3BD, while the lower band corresponded to both tUL2BD and tUL2BD [56]. In Drosophila, another tUL2-specific mAb specifically detected the lower tUL2BD/BD band, in agreement with [56], while the tUL2-specific antibody detected exclusively the upper band. (TI)

**Video S1** An exclusive anterior MT network. Video of the 3D reconstruction of the confocal stack used in Fig. 4C to reveal the MT network exclusively in the anterior half of a 1:1 embryo. (ZIP)

**Video S2** Independence of the early MT network from the actin sheet in a 1:2 embryo. Video of the 3D-reconstruction of the confocal stack used in Fig. 4C to reveal the MT network (red) is not in contact with the actin sheet (green) of a 1:2 embryo. (ZIP)

**Video S3** Independence of the early MT network from the actin sheet in a 1:6 embryo. Video of the 3D-reconstruction of the confocal stack used in Fig. 4D to reveal the MT network (red) is not in contact with the actin sheet (green) of a 1:6 embryo. (ZIP)

**Video S4** Acess and intensive MT network activity at the cortex of the tUL2/BD embryos. Video of the 3D-
reconstruction of the conical stalks used in Fig. SNC. An intact MT assembly with bright aster and a dense network is observed (red). Now that the internal nuclei (blue) are not associated with the aster. (ZIP)

Acknowledgments

We thank James Stains for providing the human placenta, Matt Righi for helpful and insightful discussions, Yuan Zhang for controlling antibodies and Mira Olgar for help with the mass spectrometry. We also thank Drs. Rekha and Mark Reddy for critical technical assistance, and Mark Nell and David Reddy for comments on the manuscript.

Author Contributions

Conceptualized the experiments, KF SB. Performed the experiments, KF MC. AC AK AH SR. Analyzed the data, KF MC. AK AH SR. Contributed to the experimental design, KF MC. AC AK AH SR. Contributed to the writing of the manuscript, KF SB.

References

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Supporting Information

Figure S1.

bcd mRNA gradients in Diptera. (A) a Drosophila embryo at fertilization hybridised with a bcd probe and alkaline phosphatase to reveal the strict accumulation of the mRNA at the anterior pole, (B) a Drosophila nc 14 embryo hybridised with a bcd probe and alkaline phosphatase showing an extended gradient, (C) a Lucilia sericata nc 14 embryo hybridised with a bcd probe and alkaline phosphatase showing an extended gradient, (D) a Lucilia sericata nc 14 embryo hybridised with a bcd probe using fluorescence. Methods and colour conversion as in Fig. 1A, B and [3].

https://doi.org/10.1371/journal.pone.0112053.s001

Figure S2.

Mislocalization of the bcd mRNA in α Tub67C and motor protein mutants. (A) a nc 1 α Tub67C1/α Tub67C1 embryo, stained for the bcd mRNA (green) along with DAPI (blue) to reveal a streak of the mRNA. (B) a nc 5 α Tub672/α Tub67C2 embryo, stained for the bcd mRNA (green), along with DAPI (blue). (C) 3-D reconstruction of the tip of a nc 5 α Tub67C3/α Tub67C3 embryo, stained with mAb YL1.2 (red) and DAPI (blue) to reveal a dense MT network and aster-like MT bundles without nuclei (asterisks). The positions of the nuclei are indicated with yellow arrows, one normal metaphase nucleus is indicated with a white arrow. A movie of this 3-D construction is available as Video S4. (D) a nc 5 Kavar21G+/+ embryo, stained for the bcd mRNA (green), along with DAPI (blue). (E) a nc 1 Kavar18C+/+ embryo, stained for the bcd mRNA (green), along with DAPI (blue). (F) a nc 3 ncd05884/ncd05884 embryo, stained for the bcd mRNA (green) along with DAPI (blue). (G) wild-type stage 10 oocyte, stained for the bcd mRNA (green), along with DAPI (blue). (H) kavarnull/DR(3L)35 stage 10 oocyte, stained for the bcd mRNA (green), along with DAPI (blue). The anterior localization is largely normal.

https://doi.org/10.1371/journal.pone.0112053.s002

Figure S3.

Specificity of Tubulin antibodies. Western analysis of 0–2 h embryonic extracts (Fig. S3) showed that mAb YL1.2 detected two Tubulin bands, in accordance with previous reports [62]. The upper band corresponded to α Tub67C, while the lower band corresponded to both α Tub84B and α Tub84D [31], mAb DM1A, another α Tubulin-specific mAb specifically detected the lower α Tub84B/D band, in accordance with [31], while the α Tub67C-specific-antibody detected exclusively the upper band.

https://doi.org/10.1371/journal.pone.0112053.s003
Video S1.

An exclusive anterior MT network. Video of the 3D-reconstruction of the confocal stack used in Fig. 1C to reveal the MT network exclusively in the anterior half of a ne 1 embryo.

https://doi.org/10.1371/journal.pone.0112053.s004

(ZIP)

Video S2.

Independence of the early MT network from the actin sheet in a ne 2 embryo. Video of the 3D-reconstruction of the confocal stack used in Fig. 2A–C. G: The MT network (red) is not in contact with the actin sheet (green) of a ne 2 embryo.

https://doi.org/10.1371/journal.pone.0112053.s005

(ZIP)

Video S3.

Independence of the early MT network from the actin sheet in a ne 6 embryo. Video of the 3D-reconstruction of the confocal stack used in Fig. 2D–F, H. The MT network (red) is not in contact with the actin sheet (green) of a ne 6 embryo.

https://doi.org/10.1371/journal.pone.0112053.s006

(ZIP)

Video S4.

Asters and intensive MT network activity at the cortex of a Tub67c3 embryos. Video of the 3D-reconstruction of the confocal stack used in Fig. S2C. An intense MT activity with huge asters and a dense network is observed (red). Note that the internal nuclei (blue) are not associated with the asters.

https://doi.org/10.1371/journal.pone.0112053.s007

(ZIP)
Figure S1

A  unf

B  14M+0min

C  14M+0min

D  14M+6min
Figure S2

A

B

C

D

E

F

G

H
Cortical movement of Bicoid in early Drosophila embryos is actin- and microtubule-dependent and disagrees with the SDD diffusion model

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Abstract

The Bicoid (Bcd) protein gradient in Drosophila serves as a paradigm for gradient formation in textbooks. The SDD model (synthesis, diffusion, degradation) was proposed to explain the formation of the gradient. The SDD model states that the bcd mRNA is located at the anterior pole of the embryo at all times and serves as a source for translation of the Bicoid protein, coupled with diffusion and uniform degradation throughout the embryo. Recently, the ARTS model (active RNA transport, synthesis) challenged the SDD model. In this model, the mRNA is transported to the cortex along microtubules to form a mRNA gradient which serves as template for the production of Bcd, hence little Bcd movement is involved. To test the validity of the SDD model, we developed a sensitive assay to monitor the movement of Bcd during early nuclear cycles. We observed that Bcd moved along the cortex and not in a broad front towards the posterior as the SDD model would have predicted. We subjected embryos to hypoxia where the mRNA remained strictly located at the tip at all times, while the protein was allowed to move freely, thus conforming to an ideal experimental setup to test the SDD model. Unexpectedly, Bcd still moved along the cortex. Moreover, cortical Bcd movement was sparse, even under longer hypoxic conditions. Hypoxic embryos treated with drugs compromising microtubule and actin function affected Bcd cortical movement and stability. Vinblastine treatment allowed the simulation of an ideal SDD model whereby the protein moved throughout the embryo in a broad front. In unfertilized embryos, the Bcd protein followed the mRNA which itself was transported into the interior of the embryo utilizing a hitherto undiscovered microtubular network. Our data suggest that the Bcd gradient formation is probably more complex than previously anticipated.
Introduction

The maternal bicoid (bcd) gene in Drosophila is described a paradigm in textbooks for gradient formation. To date, there are two prevailing models describing how the gradient is established in the first 3 hours of embryonic development. The first one, termed SDD model, after synthesis, diffusion and uniform degradation [1] states that the bcd mRNA stays at the anterior tip at all stages and that, upon translation of the protein, Bcd diffuses to the posterior, followed by uniform degradation. It is noted that diffusion has not been demonstrated experimentally, nor has it been shown in which path of the early embryo Bcd chooses to move to the posterior. In essence, the SDD model was proclaimed as a dogma without experimental evidence and has stayed in textbooks for more than two decades as a paradigm for gradient formation. The SDD model was challenged in 2009 with a report showing that the Bcd protein movement is not the cause for gradient formation, but rather the existence of a bcd mRNA gradient which serves as template for Bcd a gradient protein translation [2–5]. Later, in 2011, the SDD model was modified to the “extended SDD model” by [6] whereby a small mRNA gradient reaching about 80% egg length would contribute, but would not fully account for Bcd protein gradient formation, concluding that the major cause for gradient formation would still be Bcd protein movement.

To validate the diffusion model whose basic assumption had never been rigorously tested since its establishment in 1986 [1], fluorescent dextran particles were injected at the very tip of the embryo allowing the author to conclude that the motion of the dextran particles fits the diffusion equation well [7]. Most importantly, the particles spread uniformly over the whole inner area leading to the final conclusion that the movement of Bcd would confirm to the same diffusion properties as dextran particles.

Bcd protein distribution and posterior migration in early nuclear cycle (nc) 1–6 embryos was analyzed in detail by [8]. In addition to the posterior movement, a deep internal plume of Bcd was detected during nuclear cycles 4–6 enveloping internal nuclei, thus recapitulating the expression of the bcd mRNA in a plume at nc 4 [3, 6, 8]. This observation led [8] to propose two models of Bcd gradient formation during early nuclear cycles. The first, termed the “continuous model”, would allow a continuous redistribution of both the mRNA and the protein entirely at the cortex, while the second one, termed “2-step model”, would imply that the bcd mRNA and protein would generate an interior plume during nc 4–6, followed by the generation of a second gradient at the cortex during the blastoderm stages.

Exposure of Drosophila to hypoxia has been described in detail [9–15]. The majority of the studies were performed on adult flies, while only a few reports dealt with embryogenesis [9, 10, 16]. The first marked signs of oxygen deprivation occurred at early nuclear stages was a reversible developmental arrest, with younger embryos being more sensitive and less able to resume development than older embryos [9, 16]. An immediate reaction of internal nuclei to hypoxia was often, but not always, a characteristic condensation and movement of the DNA to the inner surface of the swollen nuclei, giving them a typical ring-like structure [9, 52 Fig]. Whether the nuclei adopted a ring-shaped configuration or not depended solely on whether nuclei arrest occurred at interphase or at metaphase where O2 deprivation was induced [16]. Another marked feature of hypoxic embryos was the fast recovery to normal development, occurring within ~10 minutes [9, 10]. All these above aspects allowed us to study Bcd protein movement in an experimental set-up ideal for testing the SDD model.

It is well known that drugs can influence bcd localization in oocytes and embryos. Most studies with drugs were performed with the intention to study the behavior of the bcd mRNA in oocytes [17, 18] and to a lesser extent also in the embryo [22]. The most commonly used drugs were those directed against the two major cytoskeletal components, the microtubules...
(MTs) and actin. To compromise the function of MTs, the MT-degrading drugs colchicine and colcemid were mostly used in the past [19]. If Drosophila oocytes were bathed in these drugs, the bcd mRNA did not localize properly to the anterior [18, 20, 21] which suggested that the MT-based transport of bcd mRNA was compromised. The drug vinblastine was shown to bind to a distinct site between Tubulin heterodimers [35], leading to the degradation of MTs, but was not used in bcd localization so far. Finally, taxol was described as a MT-stabilizing drug [19] used in the past to visualize MTs in early Drosophila embryos [22–25]. However, taxol elicited artefacts and thus did not reveal the true architecture of early cortical MTs, leading to alteration of the appearance of the anterior cortical network [5].

Actin was shown to be crucial for anterior bcd mRNA tethering at the end of oogenesis. If oocytes were incubated in the actin-depolymerizing drug cytochalasin D, bcd mRNA localization at the anterior end was compromised and stable actin-dependent anchoring of the mRNA was no longer possible [17, 20]. Likewise, the actin cytoskeleton was shown to be required for the maintenance of polar plasm components such as the nanos or oskar mRNA [26]. Other actin-targeting drugs exist [27, 28], such as the latrunculins, an actin destabilizing drug similar to cytochalasin D which was used in the past to disrupt filamentous actin (F-actin) in the early embryos [29]. Another is the F-actin stabilizing drug phalloidin which is mostly used as a tool to visualize F-actin in combination with fluorescent phalloidin-derivatives.

To monitor Bcd movement in early nc embryos, we developed a sensitive assay, coupled with the ability to apply drugs that influence Bcd movement. We demonstrate that the bcd protein migrates along the outermost cortex. Furthermore, Bcd migration is microtubule- and actin-dependent, suggesting that the Bcd gradient formation is probably more complex than previously anticipated.

Material and methods
Fly stocks
To ensure high levels of Bcd protein, the P(bcdΔ+) / PM7 stock was used (TM0, Tübingen stock list, gift of Tom Kornberg). Unfertilized eggs in larger quantities were obtained from females transgenic for the male sex peptide, P(5’-Fyp)-GFP (7:21 = Ace76/Aga, Yp1, bsc10) (P1 stock number 4365). In all cases, pre-collections were used to ensure correct age of the laid embryos. bcd mRNA patterns in those G10 embryos were indistinguishable from those of unfertilized wild type embryos.

Hypoxia and drug treatment
Embryos were collected at 25’ (using precollection) in 1 hour intervals and were exposed to hypoxia with or without drug treatment for the time indicated. The assay was as follows. After dechorionation and rinsing with tap water, embryos were transferred to a cap which was cut off from an Eppendorf vial and filled with 200 μl PBST, supplemented by 1/100 volume of embryo permeabilization buffer [52]. The solution was evaporated with a water vacuum pump to remove any oxygen, and drugs were added to the final concentration: 50 μg/mL colchicine / 20 μg/mL colcemid mixture, 10 μM taxol, 10 μM vinblastine, 20 μg/mL latrunculin B and 20 μg/mL phalloidin. Embryos were then transferred to the cap using a brush, aided by a fine forceps to remove embryos from the brush tip. All embryos sank down to the bottom despite the fact that they still harbored the vitelline membrane. Embryos were incubated for the indicate time interval in a moisture chamber at 25°C. Addition of heptane was strictly avoided as it led to a substantial increase of background during immunofluorescence. Embryos were fixed using either 4% formaldehyde (for in situ hybridization and Bcd antibody staining in Fig 1), or using heat-fixation (for all other embryos). Molecular markers in S2 Fig were Hoechst 33342,
Fig. 1. Cortical movement of Bcd in hypoxic embryos and effects of hypoxia on the segmental axis. Pictures represent 16-bit digital cortical planes of embryos oriented with their dorsal side-up and anterior to the left, except for (M-P) which show cuticles. Posterior intersegmental and the crude cortical planes were converted to a color scale with values of 0-255 (8-bit), shown in (O), except for (N) to (P) and (Q). Nomenclature of nuclear cycles follows that of [46]. (A-D) untreated Bcd^+ embryos stained with Bcd antibodies. (A) Interphase of nuclear cycle 6t (B) cycle 7 (C) cycle 8 (D) cycle 9 embryos. Note the migration of the cytoplasm at the cortex of the embryos and not to the interior. (E-H) Relative Bcd intensities of n 6 embryos in hypoxic Bcd^+ embryos and collected at different time intervals after hypoxia treatment: (E) 1-2 h; (F) 2-3 h; (G) 3-4 h; (H) 7-8 h. (I) 17-18 h. Note the movement of the Bcd protein in the “sweeping” embryos along the cortex. (J-L) Formaldehyde-fixed n 8 embryos stained with Bcd antibodies. (K-L) distribution of the Bcd mRNA in Bcd^+ embryos after 2-3 h hypoxia (K) and 3-4 h hypoxia (L). Note the sparse movement of the mRNA. Weak (M) and strong cuticle phenotype (N) of wild-type embryos 1 h after treatment with hypoxia. Embryos collected in 1 h interval, subjected to 3 h hypoxia and recovered for 3 h. Weak (O) and strong cuticle phenotype (P) of Bcd^+ embryos. (Q) 0-1 h induced, 3 h hypoxia-induced and 3 h recovery n 14 embryos, stained for Bcd protein (green) and Eve (red). Percentages indicate position of Eve stripes 1 and 7 in % egg length, respectively. Insert in (Q) shows DAPI staining of the posterior and demonstrating lack of pole cells. (R) color conversion of the Bcd pattern in (Q). (S) n 14 Bcd^+ embryos stained for Bcd protein (green) and Eve (red). Percentages indicate position of Eve stripes 1 and 7 in % egg length, respectively. (T) color conversion of the Bcd pattern in (Q).

https://doi.org/10.1371/journal.pone.0188443.g001

used at 50μg/mL Sytox Green and TO-PRO3 (Molecular Probes), used at 20 μg/mL respectively.

Antibodies and image recording

Rabbit antibodies against full-length Bcd were a gift from M. Biggin and were used at 1:500. Oat anti-Stau antibodies (Santa Cruz Biotechnology) were used at 1:200. Rat antibodies against Stau were a gift from A. Ephrussi and were used at 1:1000. mAb 1A20 directed against actin (DHIEB) was used at 1:50. All confocal pictures were recorded on a Zeiss LSM 710 at 8-bit resolution allowing 256 intensity values. Each embryo was recorded as a stack of 6-10 pictures from a start point before the middle of the embryo to an end point beyond the middle which then served as the basis to decide upon the mid-sagittal-neon section. Care was taken to ensure that the gain of all recordings was adjusted to avoid saturation of the peak intensities by adjusting the gain of the LSM 710. For color conversion and interpretation of signal intensities, the Osiris DICOM program was used [23].
Data acquisition
Intensity graphs (Fig. 2) were obtained from an ellipsoidal area moved along the dorsal cortex of mid sagittal sections, as illustrated in Fig. 3G [35]. A detailed description of used algorithms, scripts, and tools is available on request.

In situ hybridization
Fluorescent in situ hybridization (FISH) was used according to [3], except that RNA probes were used, combined with a home-made Alexa Fluor 555 Signal Amplification Kit, using identical steps as the commercially-available Alexa Fluor 568 Signal Amplification Kit (Thermo Fisher A18866).

Results
Bcd moves at the cortex and not to the interior during early nuclear cycles
To analyze Bcd movement during early nuclear cycles combined with the ability to use drugs that perturb Bcd migration, we tested numerous fixation and antibody staining protocols that would allow the combination of both approaches with an adequate signal-to-noise ratio. Since the formaldehyde fixation conditions as described in [3] constantly led to unwanted noise in combination with hypoxia and drug treatment (see below), we used heat-fusion instead. This fixation method was shown to work reliably in the past for Bcd antibody staining [3, 30]. To increase the sensitivity, we utilized a strain which produces 3 times more Bcd protein than wild type, hcd<sup>38</sup> [8, 31, 32]. Moreover, the increased levels of Bcd permitted the analysis of single confocal mid sagittal sections that allowed a precise analysis of the spatial pattern of Bcd. hcd<sup>38</sup>/embryos are known to generate a distinct Bcd gradient which differs from that of wild type embryos [1]. We monitored the distribution of Bcd in hcd<sup>38</sup> embryos in early cleavage staged embryos and used single confocal pictures derived from mid sagittal stacks. To

Fig 2. Bisect analysis of hypoxic embryos demonstrates that the extent of Bcd movement changes with time. (A-D) Bcd protein profiles of embryos at nc 8 exposed to hypoxia. (A) H2 embryos exposed to hypoxia for 1 h and shown along with a yellow line as mean profile. (B) H2 embryos exposed to hypoxia for 2 h and shown along with a green line as mean profile. (C) H2 embryos exposed to hypoxia for 3 h and shown along with a black line as mean profile. (D) H2 embryos exposed to hypoxia for 4 h and shown along with a red line as mean profile. Profiles were measured as illustrated in Fig 3G and shown as intensities with arbitrary units. (E) Superimposition of all 4 mean profiles from (A-D), shown as intensities with arbitrary units. (F) Plot of (E) shown as percentage of the total Bcd protein content at a given point of the A-P axis. (G) Overview of an embryo, illustrating positions at which Bcd protein levels were measured in overlapping ellipsoidal discs along the dorsal cortex of embryos.

https://doi.org/10.1371/journal.pone.0185443.g002
monitor the relative distribution without the necessity to graph the intensities along the A-P axis, we converted the intensities from the crude confocal file to a color scale [3, 33] allowing us to monitor the spatial distribution of Bcd. fcdb\textsuperscript{+}\textsuperscript{302} embryos produce about three times more Bcd protein than wild-type embryos (S1A, S1B, S1D, and S1E Fig.), but the appearance of the initial gradient of Bcd in fcdb\textsuperscript{+}\textsuperscript{302} embryos is retarded at nc 6 compared to an identical nc 6 wild-type embryo. This was evident from comparing the heat maps of the respective caps (S1D and S1E Fig.). This observation suggests that the system for transporting the mRNA is not scaled up to accommodate for the transport of the high amount of bcd mRNA in fcdb\textsuperscript{+}\textsuperscript{302} embryos. In a nc 6 embryo (Fig. 1A), Bcd is strongly concentrated at the tip with some posterior migration on the dorsal and ventral side, recapitulating the distribution of the mRNA, also confirmed in [8, 4] at nc 4. At nc 7, however, this distribution changes dramatically and the protein covers an anterior cap with little Bcd in the inside (Fig. 1D) and a smaller gradient extending to about 15% of the egg length. During the next two nuclear cycles (Fig. 1C and 1D), the cap with the gradient expands to the posterior reaching about 25% at nc 9 (Fig. 1D). To rule out that the heat-fixation procedure would extract Bcd protein such that only a subset of Bcd was revealed, we stained a formaldehyde-fixed nc 6 embryo (Fig 1J) that showed a staining pattern comparable to that of a heat-fixed nc 6 embryo (Fig 1A).

Hypoxia and uptake of smaller molecules
To further investigate the movement and the path of the Bcd protein along the cortex, we decided to inactivate the mRNA transportation system without affecting the viability of the embryos. We had previously shown that the bcd mRNA is transported along cortical MTs in the early embryo [5]. Hence, it would be desirable to selectively inactivate cortical MTs without affecting the interior MT network involved in nuclear division and migration, a technically-challenging scenario, as any disturbance of the function of MTs has deleterious effects on the viability of the embryo.

To overcome the above constraints, we noted that embryos exposed to hypoxia arrest growth and are virtually “sleeping”, but are readily reactivated once oxygen is supplied [9, 10]. To this end, we developed an assay to submerge embryos into water-based buffer systems rather than expose them to ascorbic in other studies [9, 10]. Our approach also allowed drugs to move into the embryo. Other drug delivery assays into embryos have been previously described [34, 35] using detergents to help to permeabilize the embryo. The disadvantage was that many of the embryos suffered from the treatment. In contrast, our simple assay allowed for the simultaneous application of any drug in the hypostatic state, despite the presence of the water-separating layer and the robust vitelline membrane [36].

To illustrate movement of smaller substances through the membranes, we assayed embryos under hypoxia by adding fluorescent substances up to a molecular weight of 1500 (S2 Fig.). During hypoxia for 2 hours and simultaneous incubation with nuclear staining tools coupled to fluorescent dyes, we were able to detect fluorescence from the DNA stain Hoechst 33342 (S2A and S2F Fig.), the DNA marker Sytox Green (S2B and S2E Fig.) and the DNA marker TO-PRO (S2C and S2F Fig.), all associated with the ring-type chromosomes (S2D and S2H Fig.). In many cases, strong fluorescence was observed at the anterior end (data not shown), suggesting that the most likely point of entry is at the micropyle, the location where the sperm enters the egg.

Bicoid movement without mRNA gradient still occurs at the cortex
We reasoned that the sleeping period would allow us to monitor the movement of the Bcd protein and to assess how fast and how far the Bcd protein can move in the time where gradient formation was predicted to occur.
We chose the 69 nuclear cycle, i.e. embryos with 32 nuclei as a reference time point to evaluate Bcd protein migration from the position where hypoxia was applied. To this end, bcd-lacZ embryos were collected during 1 hour intervals, incubated under hypoxic conditions and fixed. Only embryos at the 49 nuclear division were recorded. In 1–2 h embryos (1 hour collecting, 1 hour hypoxia, Fig. 1A), little movement of the protein was observed, compared to an untreated embryo (Fig. 1A). In 2–3 h (2 hours hypoxic) embryos (Fig. 1F), the protein migrated to about 30% egg length (EL), but still the majority of the protein remained at the tip. In 3 hours hypoxic embryos (Fig. 1G), movement continued to about 50% EL, but the bulk was still detected at the tip. In 7 hours hypoxic embryos (Fig. 1H), Bcd protein has reached about 70% EL, revealing a flat gradient. After 17 hours of induced hypoxia, some Bcd protein has reached the posterior end, barely showing a gradient. Under these conditions, we can conclude one important finding: Bicoid protein does not move throughout the whole embryo, but rather follows a discrete path along the outmost part of the embryonic cortex, as it does under normal conditions (Fig. 1A–1D).

To investigate if the bcd mRNA is the cause for the Bcd protein movement, we stained lacZ embryos that were exposed to hypoxia for the presence of lac mRNA. To account for the time that is needed to synthesize Bcd protein, about 2 minutes [3, 37], we chose hypoxic 3 h embryos, instead. In 2–3 h hypoxic embryos (Fig. 1A), lac mRNA was tightly located to the tip and very little movement was seen, consistent with its expression in wild-type embryos at a similar stage including the plume of interior mRNA [3, 6, 8]. In a 3–4 h hypoxic embryo (Fig. 1A), little change was observed and the mRNA was still located at the tip. From these two time points, we can conclude, that 1) oxygen deprivation has an impact on the localization of the mRNA, i.e. it does not move in comparison with wild type embryos, and 2) the mRNA is not the cause for the protein movement seen in Fig. 1E–1L.

Developmental consequences of hypoxia on segmental anlagen

To investigate the developmental consequences of hypoxia and the impact of Bicoid movement, we subjected wild-type Oregon-R embryos to 3 h hypoxia and allowed recovery for 36 hours in order to analyse the developmental consequences based on the cuticular pattern. The majority of the embryos (71%, S1 Table) showed an A–P axis affected, with an enlargement of the anterior segments and compression of the posterior segments, including head defects with shortened mouth hooks (Fig. 1M). A lower portion of the embryos (24%, S1 Table) showed more severe A–P axis defects with several segments missing (Fig. 1N). To compare the above effects to embryos exposed to high levels of Bcd, we monitored the cuticles of bcd-lacZ larvae. Since tcd3/lacZ is a living stock, the majority (58%) of the offspring survive without any noticeable effect on A–P axis, also noted by [31]. The remaining 42% can be divided into (39%, S1 Table), where 2 thoracic segments, T2 and T3 were lacking, associated with defects in head-involution (Fig. 1O). The remaining fraction (3%, S1 Table) contained embryos which showed a severely affected A–P axis (Fig. 1P) revealing no abdominal segments at all, while the head part was only mildly affected, revealing defects in head-involution only. This data is consistent with results from 6x tcd embryos [32]. To corroborate the cuticle defects caused by hypoxia, we stained embryos that were exposed to hypoxia for 3 hours followed by recovery for another 3 hours, with Bcd and Evf antibodies. While Bcd staining at first sight revealed a rather normal-looking gradient (Fig. 1Q) and 1D, Evf staining showed that all bands appeared stretched to the posterior (Fig. 1Q), starting from 32% for stripe 1 to 83% to stripe 7, while in untreated wild-type embryos the stripes appeared from 32% to 75% [38–40]. Most conspicuously, however, no pole cells were observed (Fig. 1Q, insert), suggesting that the fraction of posteriorly-migrated Bcd within the extra 3 hours was sufficient to suppress pole cell determination, indicative of
altered fate of posterior nuclei. In comparison, the Bcd and Eve pattern in hbcf^{bp54} embryos showed a steep Bcd gradient (Fig. 15 and 16) while Eve stripes appeared compressed, starting from 45% to 82% (Fig. 15). In contrast to the hypoxic embryos, however, hbcf^{bp54} embryos still revealed pole cells (Fig. 15, insert). We can conclude that the migration of Bcd protein during 3 hours of hypoxia causes posterior defects not associated with hbcf^{bp54} embryos. Possibly, a distinct fraction of Bcd molecules may be transported to the posterior leading to suppression of pole cell formation.

Quantitative analysis of cortical Bcd movement

To visualize cortical movement more precisely, we used the crude confocal data from larger batches of mid sagittal sections from the time intervals as seen in Fig. 1E, I, and analyzed the intensities by sliding an elliptic area along the dorsal side (Fig. 2G). In contrast to the pictures in Fig. 1, where the gain of the confocal microscope was individually adjusted to avoid saturation, the embryos of the batch series of Fig. 2 were processed, collected and stained in the same experiment, and 12 to 6 embryos from each hypoxic series were recorded in a single confocal session using identical intensity condition adjusted to the strongest signal of 1-2 h embryos. In 1–2 h hypoxic embryos (Fig. 2A), the effect of extra Bcd movement appears minimal and the gradient looks similar to that of untreated hbcf^{bp54} embryos (1). In 2–3 h hypoxic embryos (Fig. 2B), however, a portion of the anteriorly located Bcd protein has moved posteriorly, making the plot markedly flatter. This tendency continues in 3–4 h hypoxic embryos (Fig. 2C). In 7–8 h hypoxic embryos (Fig. 2D), the change of the shape of the plot is remarkably little compared to that of 3–4 h embryos (Fig. 2C) suggesting that Bcd movement has come to a halt. Possibly, the embryo suffers from the prolonged hypoxia, associated with a high degree of lethality which is inherent to young embryos [9]. When the mean curves of Fig. 2A–2D were compared (Fig. 2E), 1–2 h hypoxic embryos (yellow) revealed a distinct curve compared to the remaining 3 curves whose slopes decreased the longer the embryos were exposed to hypoxia. This observation prompted us to monitor the percentage distribution of Bcd in relation to the A–P axis after the different hypoxic incubations (Fig. 2F). After 1–2 h hypoxia, still about 80% of Bcd was contained within the first 40% of the embryos. A marked change took place in 2–3 h embryos where 80% of the protein was contained within the first 52.5% of the embryos, representing a change of 12% during one hour. In 3–4 h embryos, the value was at 57–59% indicating a reduced movement in comparison to the previous interval. Most notably, during the following 3 hours (i. e. in 7–8 h embryos), only another 5% of Bcd moved to the posterior. From this experiment, we can conclude that Bcd movement was strongest during the early phases of hypoxia, but decreased substantially during the subsequent time intervals.

Cortical movement of Bcd is microtubule-dependent

To investigate whether Bcd movement is dependent on cytoarchitectural changes of the egg, we combined our water based hypoxia assay with drug application. To this end, we added the drugs directly to the buffer for the hypoxia-treated embryos and exposed them for the time interval indicated.

[5] exposed early nc embryos to the MT destabilizing drug mixture colchicine/cocerem (CC) and could demonstrate that the anterior cortical MT network implicated in hcd mRNA transport was degraded. This data showed that drugs could enter the egg despite the water-repellent wax layer and the vitelline membrane, and that the MT network responded to the application.

Embryos exposed for 1 hour to hypoxia and CC exhibited subtle changes to the Bcd distribution pattern (Fig. 3A), compared to untreated embryos (Fig. 1E). Some protein moved more
Fig. 3. Bcd movement in hypoxia-treated embryos depends on microtubules. Pictures represent midgastrula ventral confocal planes of embryos oriented with their dorsal side up and anterior to the left. Relative intensities of the crude confocal pictures were converted to a color scale with values of 0–255 (bit). Shown in (A–C) is 6 β-actin embryos treated with colchicine (0.01% DMSO) for 1–2 h (A), 2–3 h (B) and 3–4 h (C), and stained for Bcd. (D) is 6 β-actin embryos treated with CC+T for 2–3 h, and stained for the bcd mRNA. (E) is 6 β-actin embryos treated with taxol for 1–2 h, 2–3 h and 3–4 h, and stained for Bcd. (F) is 6 β-actin embryos treated with 20 μg/ml vinblastine for 2–3 h, and stained for the bcd mRNA. Note that destabilization of microtubules compromises the cortical path in favor of a more ubiquitous path. Note that the movement in (B) corresponds to the one predicted by the ideal SDD model where the protein will move in a broad front throughout the entire embryo.

https://doi.org/10.1371/journal.pone.0185443.g003

posterily (Fig. 3A, arrows). If treated for 2–3 hours (Fig. 3B), marked changes were observed. Parts of the protein migrated to the interior of the egg and some protein entered the endoderm, an actin-rich area surrounding the nuclei (3L), as well as the nuclei (Fig. 3B, arrowheads). Of note, in CC-treated hypoxic embryos, the chromatin never formed the typical rings (3F, Fig). In embryos treated for 3–4 hours (Fig. 3C), the situation was even more pronounced with more Bcd migrating to the interior. Some Bcd even reached the nuclei in the middle of the embryo (Fig. 3C, arrowheads). To exclude the possibility that the mRNA was the cause for this movement, we stained 2–3 hours-treated embryos for the presence of bcd mRNA (Fig. 3D), which exhibited a distribution similar to that of hypoxia-only treated embryos (Fig 3B). This data demonstrated that Bcd movement was dependent on an intact MT network.

Hypoxia combined with taxol treatment revealed subtle changes in Bcd movement when exposed for 1–2 hours (Fig. 3A) compared to the reference without drugs (Fig. 1C). In 2–3 hours-treated embryos (Fig. 3B), there was little change observed except that the protein as a bulk moved to the interior (Fig. 3B). In 3–4 hours-treated embryos (Fig. 3D), some posterior Bcd movement was observed, comparable to the reference (Fig. 1C). If assayed for bcd mRNA distribution, taxol-treated embryos were almost indistinguishable from the reference (Fig. 1A). We can conclude that taxol did not have a deleterious effect on Bcd movement, nor did it promote it substantially.

Vinblastine treatment allows to simulate the SDD model

Vinblastine has been shown to affect MT growth, but has been shown to bind to a distinct site between heterodimers which are different to those by colchicine/cocemid and taxol (Florant and Mitchison, 2016). When vinblastine was applied during a 1–2 hour interval (Fig. 3D), protein movement began in all directions, and became more obvious during the 2–3 h interval (Fig. 3D). Here, the protein distributed equally over the whole inner part. In 3–4 hours-treated embryos (Fig. 3E), the situation was even more pronounced. Protein distribution moved to the posterior in a broad front, and thus seemingly conforming to the SDD model. This data allows
two interpretations: 1) Bcd protein movement requires an intact MT network in the yolk to prevent Bcd movement to the interior, 2) the cortical MT network no longer restricts Bcd protein movement to the cortex. To rule out that the cause for this massive migration being attributed to the mRNA, viroblastine-treated embryos were stained for the fcd mRNA revealing that fcd mRNA remained at the tip (Fig. 3D), as observed in CC- or taxol-treated embryos (Fig. 3O and 3Q).

Actin is indispensable for Bcd stability and cortical movement

To investigate whether actin is involved in the movement, we subjected fcd<sup>−/−</sup> embryos to drugs such as phallolidin that prevents F-actin degradation (SSA and S3B Fig), or to latrunculin B that disrupts the formation of actin bundles (SSC and S3D Fig). In phalloidin-treated embryos, long cortical microfilaments (MFs) were observed (S3B Fig), consistent with a stabilization of F-actin (S1). In contrast, latrunculin B-treated embryos revealed absence of long MFs, and only globular actin particles were detected, instead (S3D Fig), demonstrating that these drugs can efficiently alter the actin cytoarchitecture. In 1–2 hours latrunculin B-treated embryos (Fig. 4A), initial movement of Bcd was indistinguishable from the reference (Fig. 4C), while in 2–3 hours treated embryos, sparse cortical movement and fewer Bcd protein particles were observed (Fig. 4B), in comparison to the reference (Fig. 4F). In 3–4 hours treated embryos, no further movement occurred, but considerably lower amount of Bcd protein was observed, suggesting that the degradation of Bcd has commenced (Fig. 4C). As far the mRNA was concerned, fcd<sup>−/−</sup> embryos treated for 2–3 h with latrunculin B did not show any mRNA movement nor were fewer mRNA molecules detected (Fig. 4D), in comparison to the reference (Fig. 4E). This suggests that the movement and the stability of Bcd protein was not dependent on the status of the mRNA. This data proposes that an intact actin network at the cortex is critically important for both Bcd movement and stability.

When the existing actin filaments were prevented from degradation, i.e., after exposure to phallolidin, we noted that Bcd movement initially (at the 1–2 hours interval) behaved like in a wild type embryos, with the exception of a small fraction of the protein moving to the interior (Fig. 4E, arrows). In 2–3 hours exposed embryos, this behavior continued, resulting in Bcd staining in endosomal and interior nuclei (Fig. 4F, arrows). Concurrently, a greater proportion of the Bcd protein followed the cortical pathway similar to control hypoxic embryos (Fig. 4F). Longer exposure (3–4 hours interval) revealed further streaming of Bcd to the interior, and
nuclei and eggasts showed staining with Bcd (Fig. 4G, arrow), similar to embryos treated with CC (Fig. 3C). However, it was not as extensive as that seen in vitaminA-treated embryos (Fig. 3E). Again, the bcd mRNA did not move from the tip (Fig. 4H), suggesting that cortical actin does not impede movement of Bcd.

Bcd movement in unfertilized embryos is strictly dependent on the bcd mRNA which utilizes a MT pathway into the interior

It is well documented that unfertilized eggs synthesize Bcd protein and that the process of fertilization is not a prerequisite for the initiation of Bcd translation [1, 4]. A recent study monitored Bcd movement in unfertilized embryos [42] showing that the Bcd gradient appeared longer and less steep compared to fertilized embryos of the same stage. The data was then interpreted in support of the SDO model. We therefore analyzed the patterns of Bcd and Staufen protein (as a read-out system for the bcd mRNA) in unfertilized embryos during short time intervals to monitor their movement. During the first hour, there was little diffusion of Bcd (Fig. 5A) away from the source of the bcd mRNA template (Fig. 5B). During the next hour (hours 1–2), Bcd showed expansion towards the posterior, with the bulk of protein associated in a region slightly shifted posterior from the tip (Fig. 5C), congruent with the template which showed similar posterior movement (Fig. 5D). In 2–3 hours old embryos, weak and uniform Bcd covered the anterior third of the embryo likely representing freely-moving Bcd, while the bulk of Bcd stayed localized to the anterior-most 20% of the embryo (Fig. 5E), consistent with

![Fig 5. In unfertilized embryos, bcd mRNA and Bcd protein move to the interior. Unfertilized eggs collected from different time intervals, 0–1 h (A–D), 1–2 h (E–G), 2–3 h (J–L), 3–4 h (M–P), stained for Bcd protein (A, E, I, M), Staufen protein (B, F, J, N) and DAPI (C, D, K, P). The merge of all staining patterns is revealed in (D, H, L, P). Note the weak diffusion of the Bcd protein away from the bulk (A, E, I, M), which is always congruent to the Staufen protein (B, F, J, N). (Q–T) in situ hybridization of bcd mRNA at 0–1 h (Q), 1–2 h (R), 2–3 h (S) and 3–4 h (T) in unfertilized embryos. Arrows in (Q, R, S) denote last movement of the mRNA particles, apparently not associated with Staufen (compare to B, F, J). Time intervals and proteins are indicated in yellow.](https://doi.org/10.1371/journal.pone.0158443.g005)
the template (Fig 5). In late embryos (3–4 hours), Bcd showed further axial expansion of movement, including the bulk which also moved towards the interior (Fig 5A) consistent with the data of [18]. Concurrently, the Staufen protein has now migrated deeply into the interior of the embryo (Fig SN), where it showed co-localization in the bulk of Bcd (Fig 3P). Another interesting feature was that Staufen disappeared rapidly at the posterior pole, while it persisted at the anterior pole (Fig SN; SN and SN1), suggesting distinct degradation mechanisms.

Our data on Bcd protein distribution in unfertilized embryos were in agreement with data of [8, 62]. However, our data on the behavior of Staufen was in true conflict with the interpretation of the SDO model in [62] which implied that the mRNA was anchored at the anterior tip at all times. We sought to resolve this apparent discrepancy and analyzed the hcd mRNA pattern in subsequent stages of unfertilized eggs. The fragile embryos were fixed well to survive the harsh treatment during in situ hybridization. As expected and in agreement with Fig 5B, the mRNA moved away from the tip during the early stages (Fig 5Q). More importantly, subsequent stages showed that the mRNA moved to the center of the embryo as a streak (Fig 5R and S, arrows) and not to the cortex, implying that the cortical network was not established. Furthermore, the streak mRNA did not seem to involve Staufen (compare Fig 5R and S with Fig 5F and 5I), suggesting that the hcd mRNA utilizes another adapter protein than Staufen for the transport along the streak. At late stages (3–4 hours), hcd mRNA encountered a barrier (Fig 5T, arrowhead) which prevented further transport towards the posterior. Our data demonstrates the presence of an internal MT network in unfertilized eggs, which serves as a path for migration of the mRNA, however, the cortical network is only built up upon fertilization [5]. Occasionally, in about 1–2% of fertilized wild-type embryos, a thin mRNA streak was observed, where the direction of the streak was always pointing towards the dividing nuclei (unpublished).

Discussion

The present study sheds light onto the movement of the Bcd protein and demonstrates that the Bcd protein follows a discrete pathway at the cortex during early cleavage stages. The movement of Bcd during the early stages was never investigated experimentally until recently [18], but rather relied on assumptions based on the dogma of hcd gradient formation [1], the proposals of the SDO model, and on the simulation of protein movement by injecting dextran particles [2].

Several assumptions were put forward in the past [6, 43–65] that should explain the apparent paradox of a diffusion coefficient of Bcd which was two orders of magnitude too low. Summarized in [64] were: 1) diffusion might be much higher in other compartments, for example, within the center, 2) diffusion might be faster during nuclear cycles, i.e., at a time when Bcd could not be observed by live imaging. 3) several fractions of Bcd molecules exist that have different diffusion properties. 4) active transport of Bcd.

For the first two assumptions, our data provides evidence that neither assumptions mentioned above can account for the diffusion properties as they were revealed. This for the following reasons: 1) the underlying cause for the protein gradient is the mRNA gradient [2, 3], 2) the protein moves along the cortex (this report).

The yolk, a non-permissive-territory for Bcd movement

The cortical movement of Bcd in wild-type embryos revealed that the inner yolk mass was a non-permissive territory for Bcd movement (Fig 1). In the past, the yolk was considered to be a compartment where Bcd could diffuse fast enough to explain the apparent low diffusion constant [43]. Our data in Fig 1 refutes this possibility. In line with our observations for a distinct
regionalization of Bcd movement, [46] described the inner yolk mass as an ellipsoid entity which morphologically behaved as a distinct unit during each syncytial nuclear cycle, while the cortex was described as a completely different entity.

Data about the content and structure of the inner yolk is sparse, mostly due to the inability of the laser of a confocal microscope to penetrate the dense yolk layer. Actin microfilaments in the deep yolk were described [47, 48], but require a more detailed description. As far as MTs are concerned, only the spindle apparatus during the nuclear cycles were bright enough to become visible [5].

Our drug treatment data demonstrated that the yolk became permissive for Bcd if embryos were treated with CC, viabllastin (Fig 3) or with phalloidin (Fig 4). The most pronounced of these cases was with viabllastin-treated embryos (Fig 3B–3K). The protein behaved exactly as the SOD model would have predicted; it moved to the posterior in a broad front (Fig 3K), while the mRNA remained at the tip.

In unfertilized eggs, the Bcd protein was also found in the yolk, but this observation was not the result of movement from the cortex, rather it represented de novo synthesis of Bcd from the template which was translational to the yolk (Fig 3). During longer incubations, we observed some movement of the protein away from the source (Fig SI and SM), indicating that the yolk became “permissive” for Bcd movement under these conditions. Possibly, in a unfertilized embryo, the components of the yolk alter with time, making it permissive for Bcd.

The role of actin for Bcd stability and movement

In the past, it was shown that actin was instrumental in anchoring bcd mRNA to the anterior of stage 14 oocytes [17, 20], while only sparse information was available on actin’s role for the Bcd protein in the early embryo.

For the first time, we could demonstrate that actin has a profound impact on Bcd stability and movement in the embryo (Fig 4). Particular actin-dependent, Bcd-stabilizing function (Fig 4A–4C), may constitute an undiscovered means to fine-tune Bcd gradient formation and nuclear filling, as actin is predominant at the cortex where gradient formation actually takes place. We noted that Bcd was particularly enriched around the energids under circumstances where actin was stabilized, i.e. upon phalloidin treatment (Fig 4F and 4G). During MT-drug treatment, i.e. when actin function was not compromised, a similar enrichment of Bcd in energids was observed (Fig 3B, 3C, 3I and 3K). This observation suggests that Bcd tends to accumulate in territories where there is high levels of actin. Similar observations were made by [49]. Possibly, the energids could serve as a reservoir for Bcd to quicken the step of nuclear filling after the nuclear membrane has formed again.

In cases, where F-actin was destroyed (Fig 4A–4C, 3I and 3K Fig), we also noted a second function of F-actin aiding Bcd movement to the posterior. While degradation could contribute to a general picture that there was less posterior migration due to a lack of signal, increase of the gain during the confocal analysis confirmed that Bcd movement is indeed imputed (not shown). While actin-dependent long-range movement of Bcd is an unlikely process to occur in wild-type embryos, it suggests that actin may play a role for short-range movement of Bcd, e.g. from the site of synthesis to the energids or directly to the nucleus.

Interior transport of bcd mRNA

In unfertilized eggs, the cortical MT network for bcd mRNA transport was not activated [5] and hence the bcd mRNA was not transported along the cortex (Fig 3). Instead, the mRNA was transported, presumably via MTs in the yolk into the middle of the embryo. Concomitantly, the Bcd protein was translated and was confined to the interior of the egg (Fig 3A, 3E).
S1 and SM). Although we could confirm the data of [42], i.e., that a longer, shallower Bcd gradient was observed compared to fertilized embryos of the same age, we could not support their claim that the SDD model was the basis for their observation. Our results demonstrated active transport of the mRNA into the interior of the egg, followed by translation [1]. The weakness of the conclusions drawn by [42] was that they were based on the assumption that the mRNA would stay anchored at the tip which was not the case (Fig S2–S3). Our observations were not consistent with an involvement of the SDD model to explain the apparent extended range of Bcd diffusion. Another striking observation was that the inward migration of the mRNA in unfertilized embryos was faster than cortical transport in fertilized embryos (Fig S8 and S9), raising the possibility that 1) either the tubulin composition of the two MT networks was different from that at the cortex; 2) the interior MT network was oriented more parallel to the A–P axis than the cortical one; 3) the internal MT persisted during all nuclear cycles.

Conclusions

Why does localization and movement of the Bcd protein at the cortex make sense? In the past, we and others have shown that the mRNA gradient follows a similar discrete cortical path as the protein [2, 3, 5, 8]. Hence, the protein as a direct consequence of its template is located in close proximity to the mRNA. Since the egg undergoes strong periplasmic contractions during each nuclear cycle [46], any protein gradient generated by free movement from the tip would suffer from unwanted turbulence. However, since the information of the gradient is already stored at the level of the mRNA [2, 3, 5], the mRNA associated with microtubules is much more resistant against cytoplasmic turbulences than the freely floating Bcd protein. Another feature is that local Bcd synthesis and the shuffling activity of Bcd between the periplasm and the nucleus might be under intricate control. Reported as a rather intriguing result, the concentration of Bcd in nuclei after every mitosis is surprisingly constant, taking into account that number and volume of nuclei vary with each nuclear cycle [63]. Here, we wish to add a rather simple explanation for this seemingly intriguing result: Since each point along the A–P axis experiences an increase of Bcd mRNA concentration during each nuclear cycle, the number of templates for the translation of Bcd is adjusted for the need of each nuclear cycle. This model could be extended by proposing that during each nuclear cycle, the Bcd protein could be degraded, facilitated by the presence of the PEST domain [45, 56, 51]. This model has barely been taken into consideration in the past, except in [43]. De novo synthesis of Bcd takes typically 2 minutes [3, 37], permitting enough time to fill the nuclei with the correct amount of Bcd, based on the mRNA template. Moreover, since Bcd is translated locally, we envision that there might be tightly adjusted control mechanisms linking the process of translation to nuclear translocation, thereby allowing to adjust intricately the concentration of Bcd within the nuclei.

Supporting information

S1 Fig. Comparison of Bcd patterns of bcd<sup>−/−</sup> and wild-type embryos. (A), (D), (G) nc 6 bcd<sup>−/−</sup> embryo stained for Bcd (A) using saturation of intensities at the tip, color conversion (D) from 0–255 (8 bit), and Stau2n (G). (B), (E), (H) nc 6 wild-type embryo stained for Bcd (B) and analyzed using identical confocal parameters as for (A), color conversion (E) with a maximum scale of 1/3 of (D), i.e., from 0–85, and Stau2n (H). (C), (F), (I) identical nc 6 wild-type embryo as in (B), recorded using confocal parameters for saturation of intensities at the tip, color conversion (F) with maximum scale of 255, and DAPI to reveal nuclei (I). Note that bcd<sup>−/−</sup> embryos express about 3 times more Bcd than wild-type embryos, best seen at color conversion between (D) and (E). Likewise, Stau2n as a read-out for bcd mRNA is equally
stronger in hcr^{-/-} embryos (G), compared to wild type embryos (H).
(TIF)

S2 Fig. Movement of small molecules into Drosophila embryos. (A-D) nc 8 embryo exposed for 2 hours to hypotica, simultaneously incubated with 3 molecular markers and recorded as a single stack in different channels, revealing Hoechst 33342 (A), Sytox Green (B), TO PRO3 (C) and merge of (A-C) in (D). (E-H), high magnification of a single stack recording of the embryos in (A-D) showing Hoechst 33342 (E), Sytox Green (F), TO PRO3 (G) and merge of (E-F) in (H). Note the condensation of the chromatin under hypotica conditions at the inner surface of nuclei, first described by [9].
(TIF)

S3 Fig. Effect of phalloidin and latrunculin B on cortical actin microfilaments. (A-D) nc 6 embryos exposed for 2 hours to hypotica and to phalloidin (A, B) and to latrunculin B (C, D). (A) sagittal confocal section, actin staining as revealed with mab JL20 (red) together with DAPI (blue); (B) cortical confocal section of the anterior tip at high magnification of the same embryo as in (A), stained for actin (red). (C) sagittal confocal section, actin staining as revealed with mab JL20 (red), together with DAPI (blue). (D) cortical confocal section of the anterior tip at high magnification of the same embryo as in (C), stained for actin (red). Note the extended actin microfilaments upon phalloidin-treatment (B), compared to the globular actin appearance upon latrunculin B-treatment (D).
(TIF)

S1 Table. hcr^{D-/-} phenotypes. Percentages of cuticular phenotypes of 3 h hypotica and 36 h recovered embryos (left) and hcr^{D-/-} embryos (right). 3 classes were compared, normal cuticle (blue), mild cuticle phenotype (red) and severe cuticle phenotype (green).
(TIF)

Acknowledgments
We thank Anne Ephrussi for providing rat anti-Sas6 antibodies, Mark Piggins for providing rabbit anti-Bicoid antibodies, Tom Kornberg for the hcr^{D-/-} stock and Catarina Rippe for the gift of latrunculin B. We also thank Sol Da Rocha for excellent technical assistance and Linda Wei and Thomas Blom for comments on the manuscript.

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References


Supplement Figure 1. Comparison of Bcd patterns of bcd+5+8 and wild-type embryos.

(A), (D), (G) nc 5 bcd+5+8 embryo stained for Bcd (A) using saturation of intensities at tip, color conversion (D) from 0-255 (8 bit), and Staufen (G). (B), (E), (H) nc 5 wild-type embryo stained for Bcd (B) and analyzed using identical confocal parameters for (A), color conversion (E) with a maximum scale of 1/3 of (D), i.e. from 0-85, and Staufen (G). (C), (F), (I) identical nc 5 wild-type embryo as in (B) recorded using confocal parameters for saturation at tip, color conversion (E) with maximum scale of 255, and DAPI to reveal nuclei (G). Note that bcd+5+8 embryos express about 3 times more Bcd than wild-type embryos, best seen at color conversion between (D) and (E). Likewise, Staufen as a read-out for bcd mRNA is equally stronger in bcd+5+8 embryos (G), compared to wild-type embryos (H).

Supplement Figure 2. Movement of small molecules into the Drosophila embryo.

(A)-(D) nc 7 embryo exposed for 2 hours to hypoxia, simultaneously incubated with 3 molecular markers and recorded as a single stack in different channels, revealing Hoechst 33342 (A), Sytox Green (B), TO-PRO3 (C) and merge of (A-C) in (D). (E)-(H), high magnification of a single stack recording of the embryo in (A-D) showing Hoechst 33342 (E), Sytox Green (F), TO-PRO3 (G) and merge of (E-F). Note the condensation of the chromatin under hypoxic conditions at the inner surface of nuclei, first described by [13].

Supplement Figure 3. Effect of phalloidin and latrunculin B on cortical actin microfilaments.

(A-D) nc 6 embryos exposed for 2 hours to hypoxia and to phalloidin (A, B) and to latrunculin B (C, D). (A) sagittal confocal section, actin staining as revealed with mab JLA20 (red) together with DAPI (blue). (B) cortical confocal section of the anterior tip at high magnification of the same embryo as in (A), stained for actin (red). (C) sagittal confocal section, actin staining as revealed with mab JLA20 (red), together with DAPI (blue). (D) cortical confocal section of the anterior tip at high magnification of the same embryo as in (C), stained for actin (red). Note the
extended actin microfilaments upon phalloidin-treatment (B), compared to the globular actin appearance upon latrunculin B-treatment (D).

Table 1.

Percentages of cuticular phenotypes in 3 h hypoxic embryos (left) and bcd+5+8 embryos (right). 3 classes were compared, normal cuticle (blue), mild cuticle phenotype (red) and severe phenotype (green).
Figure S1

Figure S2
Segmentation gene expression patterns in *Bactrocera dorsalis* and related insects: regulation and shape of blastoderm and larval cuticle

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ABSTRACT The oriental fruit fly, *Bactrocera dorsalis*, is regarded as a severe pest of fruit production in Asia. Despite its economic importance, only limited information regarding the molecular and developmental biology of this insect is known to date. We provide a detailed analysis of *B. dorsalis* embryology, as well as the expression patterns of a number of segmentation genes known to act during patterning of *Drosophila* and compare these to the patterns of other insect families. An anterior shift of the expression of gap genes was detected when compared to *Drosophila*. This shift was largely restored during the step where gap genes control expression of the pair-rule genes. We analyzed and compared the shapes of the embryos of insects of different families, *B. dorsalis* and the blow fly, *Lucilia sericata* with that of the well-characterized *Drosophila melanogaster*. We found distinct shapes as well as differences in the ratios of the length of the anterior-posterior axis and the dorsal-ventral axis. These features were integrated into a profile of how the expression patterns of the gap gene *Kniregor* and the paired-rule gene *even-skipped* were observed along the A-P axis in three insect families. Since significant differences were observed, we discuss how *Kniregor* controls the even-skipped stripes. Furthermore, we discuss how the position and angles of the segmentation gene stripes differ from other insects. Finally, we analyzed the outcome of the expression patterns of the late acting segment polarity genes in relation to the skeleton of the naked-cuticle and denticle belt area of the *B. dorsalis* larva.

KEY WORDS: *Bactrocera dorsalis*, segmentation, *Kniregor*, even-skipped

The oriental fruit fly *Bactrocera dorsalis* (Meigen) (Diptera: Tephritidae) is a major cause of damage to fruit production in tropical and sub-tropical regions, often leading to a total failure of crop production (Vargas et al., 2015). It has in fact been classified as belonging to the most destructive category of insects (category A pests) (Vargas et al., 2015). *B. dorsalis* is widely prevalent in many countries across Asia, but also in the U.S. state of Hawaii. Since accidental introduction in the 1940s, *B. dorsalis* has established itself as a common pest. The insects have also occasionally been detected in the U.S. mainland, e.g. in California and Florida. Attempts have been made to eradicate the pest during four major outbreaks between 1960 and 1967. In Asia, eradication was not possible for monetary reasons initially. Consequently, damage on crop production was so severe that eradication programs were ultimately impractical to combat this insect.

Phylogenetically, Tephritidae is a family of fruit flies, located immediately adjacent to that of Drosophilidae (Yang et al., 2018). Both families belong to the sub-section Acalyphina, and are referred to as “fruit flies” while the Drosophilidae are often referred to as the “common fruit fly.” To make Tephritidae more distinct from Drosophilidae, they are often called “medicinal flies.” Both families maintain a similar body shape, however, Tephritidae are usually larger than Drosophilidae and are often more colorful with pictured wings. The third family that will be described below is Calpophilidae, a family that is phylogenetically even closer to Tephritidae than to Drosophilidae (Anders et al., 2016). Within the Calpophilidae, we will focus on the blow fly, *Lucilia sericata*.
Within the family of Tephritidae, several other insect members have been described to some detail such as Ceratitis capitata (the Mediterranean fruit fly) (Stocker et al., 2014) and B. oleae (the olive fly) (Menegaz-Teixidou, 2002). Common to this family is that all these fly species represent the most destructive of fruit pests. Ceratitis capitata, in particular, because of its wide distribution over the world, its ability to cause considerable damage to fruit and its relatively smaller size, is recognized as one of the world's most economically important fruit fly species.

The body of the adult B. dorsalis is about 8 mm in length, with a wingspan of about 7 mm. Its body color is quite variable; the thorax is mainly dark brown with yellow stripes, white on the abdomen; the abdomen is light brown with two black stripes parallel to the anterior-posterior (A-P) axis, and a black stripe along the midline. More than 160,000 kinds of fruits and vegetables are known to be attacked by B. dorsalis. Mango, papaya and avocado are the principal food sources (Saliani et al., 2012).

Embryogenesis and the formation of segments in insects follows two modes: in short-germ-band development, segments are added sequentially by adding them to the posterior of a growing embryo (reviewed by Davies and Patel, 2003). The blastodermic embryo occupies only a small fraction of the egg (the remainder consists of yolk and extra-embryonic tissues). Anterior segments are already determined during the blastodermal stage, while posterior segments are added only after gastrulation. In contrast, in insects using long-germ-band development (where B. dorsalis belongs to), the body axis is established already during oogenesis and segments are formed through subdividing the embryo into equally-sized subdomains (Davies and Patel, 2003). Most long-germ-band embryos take up a large proportion of the egg, and segments are already determined before gastrulation begins. No tissue growth is involved in this process. The morphological formation of segments occurs much later in development; segmental boundaries are visible only at the extended germ-band stage.

In Drosophila, the process of segmentation was described in detail and revealed that at the macular level, a hierarchy of segmentation genes was crucial for setting up the anterior-posterior axis (Paul et al., 2001). Since segmentation is the common denominator of all insects, attempts have been made to link developmental and functional studies to show that the same common molecular models of regulation and to explain differences that were observed. The best defined gene regulation module is the gap/gene network model, which is based on the notion that a common set of regulatory modules is conserved across species, and the differences that led to the identification of important genes involved in the sexual development of B. dorsalis females, which consequently may help to develop sterile programs (Cheng et al., 2015). Concretely, a draft sequence of a relative of B. dorsalis, B. tryoni, was made available in 2014 (Glaub et al., 2015). Additional information on the embryology and development of B. tryoni is available with the upcoming sequence (Singh et al., 2016) and that of another Tephritidae, B. tryoni (Anselmo, 1964).

To supplement the information currently available in describing B. dorsalis embryology, we have characterized its embryonic development in detail. We describe the embryonic development of this species, present a panel of expression patterns of early segmentation genes and compare those to the known expression patterns of other established model insects. To address the question whether or not the control of the pair-rule genes by the gap gene is conserved between these insect families, we superimposed the expression patterns of the gap gene Krüppel and those of the pair-rule genes in B. tryoni.
rule gene even-skipped and draw conclusions how Knüppel can control the even-skipped stripes in the different insect families. We also noted that the angle and behavior of the even-skipped stripes are distinct from those of previously characterized insects with respect to the A-P axis. Furthermore, we show that the shape of the B. dorsalis egg and the behavior of the nuclei during the blastoderm stage is considerably different from that of previously characterized insects. Lastly, we analyzed the outcome of the expression patterns of the late segment polarity genes in relation to the arrangement of the naked-cuticle and denticle belt area of the B. dorsalis larva.

Results

Embryogenesis of B. dorsalis

To date, only sparse information of B. dorsalis embryogenesis and postembryonic development exists. Some information exist for two closely-related species, B. tau (Sanghi, 2006) and B. ryowi (Anderson, 1964), respectively. For this reason, we have analyzed and summarized relevant stages of the B. dorsalis life cycle in Fig. 1. We refer to the nomenclature of the stages of Drosophila embryogenes as previously described (Campbell-O’Grady and Hartenstein, 1985) and also those of the blow fly Lucilia sericata (Mellenrin et al., 2006, Bleichert et al., 2011). Embryonic development of B. dorsalis proceeds as a long germinal band (Davis and Patel, 2002). As in most insects, the egg is surrounded by a vitelline membrane and a chorion, however, in contrast to Drosophila, no dorsal appendages are observed. Once the chorion is removed, an egg is revealed which is about 0.8 mm long and 0.3 mm in diameter (Fig. 1). The ratio of the length to width, however, is quite different from that of Drosophila rendering the egg prone to pressure and physical manipulations. The ventral side as well as the dorsal side are usually curved, a feature not found in Drosophila. The posterior tip is often pointed (Fig. 10) giving the egg a distinct form, compared to those of Drosophila or Lucilia (Fig. 7B).

After fertilization, the first nuclear cleavage is very similar to those of Drosophila where the nuclei divide in the interior of the yolk (Fig. 1 A,B) until they migrate to the periphery to form a syncytial blastoderm (Fig. 1B). In contrast to Drosophila where the pole cells (the future germ cells) are formed at nuclear cycle (nc) 10 at the posterior pole, the pole cells are only formed at nc 12 in B. dorsalis (Fig. 1C). As in Drosophila, after nc 10, four more nuclear cycles follow until the stage of cellular blastoderm is reached (Fig. 1 C,D). Interestingly, during nc 14, no elongation of nuclei occurs as is observed in Drosophila, hence nuclei remain rather round during this nc (Fig. 1C, D). At about 6 hours after egg deposition, the first signs of gastrulation become obvious (Fig. 1E). However, the shape of the cells still does not change and they remain round. Anteriorly, a prominent circumferential furrow is observed which corresponds to the epiblast furrow in Drosophila. Ventradly, the mesoderm starts to invaginate (Fig. 1E). On the lateral side, up to 4 folds are observed. This is due to the fact that the radius of the

Fig. 1. Embryogenesis and imaginal discs of B. dorsalis. (A-D) E20 embryos are oriented anterior to the left and dorsal side up, unless otherwise noted. (A-D) DAPI staining. Nuclear cycles (n) are indicated in yellow. (A) Nuclear cycle 0: 1 embryo staining showing the polar body (p) and the zygotic nucleus (zn). (B) nc 0.5 embryo. Note absence of pole cells. (C) nc 12 embryo. Note the presence of the pole cells (p). (D) nc 14 embryo showing the pole cells (p) and a small number of cells at the posterior tip. (E) nc 14 showing the pole cells (p), 2-4 bright yellow microspores. (F) Gastrulating embryo showing the epiblast furrows (f) and up to 4 posterior transverse fold (v). Anteriorly, an anterior fold is observed (d). (G) Embryogenesis during germ band retraction. (H) Gastrulating embryo showing the epiblast furrows (f) and up to 4 posterior transverse fold. (I) Dark field picture of a 1st molar larva with amputation of the segments and segmental borders in yellow. The ratio of the distance of naked cuticle versus denticle belts is indicated above A3. Abbreviations: mh, mouth hook; mt, malphigian tubule; f, fold.
curve leading to germ band extension is smaller in Bactrocera than in Drosophila with respect to the dimension of the body axes. 10 hours into development, the germband is fully extended (Fig. 1F), and its shape appears quite similar to that of Drosophila or Locusta. At approximately 14 hours of development, the germband starts to retract (Fig. 1G) taking about 10 hours to complete retraction (Fig. 1H). The following morphogenetic movements such as head involution or dorsal closure proceed as they do in Drosophila, however, the speed by which they progress is considerably slower, as noted by (Vargas, 2000; Anderson, 1994). Only after approximately 48 hours at 25°C, the larvae hatch (Fig. 1K) showing an identical number of segments compared to Drosophila or Locusta. A head, 3 thoracic segments, 8 abdominal segments and a tail (Jurgens, 1987; Martinez Arias, 1993; Mellonhann et al., 2006). The patterns of the denticle bands are distinct from that of Drosophila (Moliner et al., 1999) or that of Locusta (Mollenhann et al., 2006), showing a gap between weaker anterior rows and stronger posterior rows (Fig. 1K), the latter caused by thicker individual denticle hairs. The ratio of the distance of naked outside to that of the denticle bands was about 2:1, a feature distinct from other insects (Fig. 1K; Mollenhann et al., 2006).

B. dorsalis undergoes 3 larval stages, as does Drosophila and Locusta. The larval stages last about 8 days at 24°C (Vargas, 2000). These values are also larger than those of Drosophila or Locusta. At the end of the 3rd instar larval stage, B. dorsalis larvae show a peculiar behavior inherent to most aphid-like larvae. Their body is elongating and releasing the larva, which emerges from the puparium and is able to move. The pupal stage lasts about 12 days at 24°C (Vargas, 2000). Hence, the complete life cycle of B. dorsalis can take as long as 39 days at 24°C (Vargas, 2000), 40-50% longer than in Drosophila, mostly due to the prolonged larval and pupal stage.

To determine if B. dorsalis utilizes imaginal discs to form the precursors for the adult structures, 3rd instar larvae were dissected to look for conspicuous discs such as wing and eye-antennal discs to investigate if they resemble those of Drosophila (Fig. 1), J). A wing disc is similar in shape to that of Drosophila (Fig. 1) and so is an eye-antennal disc (Fig. 1D). Photoreceptor cells follow a similar developmental fate as in Drosophila or Locusta, as revealed by the expression of a morphogenetic furrow (Fig. 1L). Altogether, this data demonstrates that imaginal disc development of B. dorsalis and probably that of most aphid-like larvae resembles strongly that of Drosophila or Callophila.

D. dorsalis segmentation genes

In order to analyze Bactrocera segmentation gene expression, we first screened a database (Galet et al., 2014) for the presence of orthologous segmentation genes of the prime model system Drosophila melanogaster. In Drosophila, the process of segmentation was described in detail showing that a hierarchy of segmentation genes is crucial for setting up the anterior-posterior axis (Pfeil et al., 2005). In Bactrocera, all genes of this hierarchy were found (Galet et al., 2014). For example, the maternal gene zicoid (zic), shown to be a feature of higher Diptera only, is not present in the Bactrocera genome. In Drosophila, bcd/oscies was expressed in a protein gradient along the A-P axis (Chever et al. and Nöelchen-Volhard, 1988), preceded by the formation of a mRNAs gradient (Fgriger et al., 1989; Sperl et al. 2009; Fahmy et al., 2014). The protein gradient serves as morphogenetic gradient to pattern the anterior-posterior axis.

In insects where zicoid is lacking, it was proposed that two genes described in Drosophila as gap genes, the hunchback (hb) and the orthodenticle (otd) act cooperatively to pattern the anterior-posterior axis, instead (Pfeil et al., 2005; Scherle et al., 2009).

With this in mind, we have analyzed the expression patterns of important Bactrocera members of the segmentation gene hierarchy (Nöelchen-Volhard and Wieschaus, 1985). Of the classes of genes at the top of the hierarchy, we analyzed the strand of genes, followed by a member of the gap gene class, the Krüppel (Kruppe) gene. The pair-rule gene class is represented by the even-skipped (eve) gene, while the segment polarity gene class by the engrailed (eng) gene.

Fig. 2: Embryonic expression of B. dorsalis hunchback (Hb). (A-E) All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted. The pteronale is in a broad anterior domain. (A) Early cleavage: stage embryo, no maternal expression is detected. (B) Early nc 14 embryo, an additional posterior band emerges. In the anterior broad band, the posterior end increases transcription (arrowhead). (C) Late nc 14 embryo, the anterior end of the broad domain loses transcription (arrowhead). In the posterior part, a distinct band becomes visible (arrow). (D) Early gastrulation embryo, the broad anterior band has resolved into a dorsal band (arrowhead) and two adjacent bands (arrow). While the posterior bar shows a dorsoventral restricted expression (arrowhead) and the midline strong posterior band (E) Gs96 ectoderm, a repression pattern of ectodermal at the midline, (F) Extended germ band, transcriptions are in a segment-specific pattern in specific neuroblasts. (G,H) Gs96ectoderm repressed embryo, lateral (G) and ventral (H) views respectively, a repetitive segmental pattern in neuroblasts is observed. (I) Late embryogenesis, transcription pattern mainly in the ventral chord.
Expression of hunchback (hb)

Expression of Bactrocera hb commences at nuclear cycle (nc) 13 where a broad domain extending was detected from about 5% to 50% egg length (EL), where the anterior tip is defined as 0% and the posterior tip as 100% (Fig. 3A). In Drosophila, this expression is similar, although the anterior domain included the whole anterior tip (Bender et al., 1989; Tautz and Pfeifle, 1989; FlyBase). In contrast to Drosophila, however, no obvious maternal hb contribution in Bactrocera was detected during the early nuclear stages (Fig. 3A). At early nc 14, the posterior end of the broad hic domain showed increased levels of transcription (Fig. 3B, arrow), while at the posterior end a posterior stripe appeared. During later stages of nc 14, the anterior end of the broad domain showed decreased levels of hb (Fig. 3C, arrowheads) and the posterior band increased in intensity. At early gastrula (Fig. 3D), expression of the anterior domain almost completely ceased, with the exception of two distinct stripes in the middle (Fig. 3D, arrow) and an anterior dorsal patch (Fig. 3D, arrowhead). The posterior band remained strong with the anterior end showing expression only dorsally (Fig. 3D, asterisk). In Drosophila, a similar pattern was observed (Astrid et al., 2016), however, only one stripe was found in the middle and the stripe was shown shifted to the anterior, in comparison to Bactrocera. During germ band extension (Fig. 3E), a repetitive pattern of hb stripes evolved, with the posterior-most expression showing strongest expression. At extended germ band (Fig. 3F), strong expression was observed in neuroblasts in a segmented pattern. During germ band retraction (Fig. 2G-I), hb expression was observed in neuroblasts. During late embryogenesis (Fig. 3G), many neurons in the ventral nerve cord showed strong hb expression.

Expression of orthodenticle (otd)

Expression of otd initiated as a broad circumferential anterior domain at early nc 14, ranging from 2% to 34% EL (Fig. 3A) and leaving the tip free (Fig. 3A, arrowhead), thus clearly later than B. dorsalis hb (Fig. 2A) and making otd a possible target of regulation by hb. During nc 14 (Fig. 3B), the anterior edge shifted to about 11% EL (Fig. 3B, arrowhead), while the posterior edge remained...
constant. During early gastrula (Fig. 3C), transcripts disappeared further from the anterior edge (Fig. 3C, arrowhead) and comprised a band from 17-34% EL. At the ventral side, the presumptive mesodermal anlagen was devoid of any transcripts (Fig. 3C, arrow), similar to that in Drosophila (Finkelstein et al., 1990; Athirai et al., 2016). During gastrulation extension (Fig. 3D), the anterior domain remained. At extended germband (Fig. 3 E,F), a two-cell-wide stripe with lateral extensions emerged (Fig. 3, arrow). These cells correspond to the midline cells that mark the outermost cells of the invaginating mesoderm. A similar expression in midline cells was also observed in Drosophila (Finkelstein and Parrishon, 1990; Athirai et al., 2016). However, while transcription of odd continuous in Drosophila, odd transcripts in B. dorsalis ceased after the extended germband stage. Contrary to our expectation, we noted that in two other Thysanurans, the medfly Ceratitis capitata and the carabid Austenaphis suspensa, maternal odd expression was observed (Schmale et al., 2008). Moreover, medfly odd showed a wide band of expression at the early blastodermic expression, and only at cellular blastoderm, all three Thysanuran cell patterns converged to an identical broad anterior band of about 17-34% EL. Therefore, there is considerable plasticity of odd gene regulation between close relatives within the same family, also noted by (Schmale et al., 2008).

Expression of Krüppel (Kr)

Kr expression in B. dorsalis started with a broad band in the middle of the embryo from about 30% EL to 75% EL, with tapered expression on either side at 1/3 (Fig. 4A). Its occurrence paralleled that of hth (Fig. 2A). At early nc 14 (Fig. 4C), a band from 45% to 60% with sharp anterior and posterior boundaries emerged, while anteriorly, a new band appeared whose width tapered off on the ventral side (Fig. 4C, arrowhead). At early gastrula stage (Fig. 4D), a posterior band appeared and the broad middle band began to split (Fig. 4D, asterisk). Slightly later, at the anterior end, another band appeared (Fig. 4E, marked with +), while the initial anterior bands broadened and segregated.

Fig. 5. Embryonic expression of B. dorsalis even-skipped (eve). (A-N) All embryos are oriented anterior to the left and dorsal side up, unless otherwise noted. (A) Early nc 14 embryo, eve is expressed in a broad central domain with diffused eve in the anterior band is observed, along with further weak bands (arrowheads). (B) Mid nc 14 embryo, the weak bands become stronger and anteriorly another broad band appears, forming bands 1, 2, 5 and 6 (B). Mid nc 14, slightly older than (C), shows band 6 with different widths and intermediate evens. Bands 6-14 have finer width and position, bands 1-5 are still determined. Numbering of stripes according to the final 7 stripe pattern in (F). (E) Nc 14 embryo close to completion, all bands are established, band 3 is considerably weaker. (F) Early gastrula embryo. 7 bands with equal intensity have evolved. (G) Embryo at beginning of germband extension, posterior to band 7, bead 6 appears. Bands 1-7 become weaker. (H) Extended germband embryo, only the posteriormost band 8 remains. (I) Embryo at beginning of germband extension, posterior band is still visible. Single cells, presumably in tracheal precursors (B), start to express eve. (J) Germband retracted embryo, a repetitive pattern in certain neuroblastoids and tracheal cells (B) is observed. (K) Ventral of a similar staged embryo as in (J), expression is conspicuous in neuroblastoids and tracheal cells. (L) Late embryogenesis, eve expression is in the ventral nerve cord in neuroblasts and the posterior germline (P). (M) Confocal picture of a late nc 14 of a D. melanogaster embryo. Eve protein expression is in green, along with DAPI-Blue to reveal the nuclei. The angle of the 7 stripes with respect to the 4-x axis is indicated in shaded green. (N) Confocal picture of a late nc 14 L. baccata embryos. Eve protein expression is in green, along with DAPI-Blue to reveal the nuclei. The angle of the 7 stripes with respect to the 4-x axis is indicated in shaded green.
The band in the middle divided into 3 bands (Fig. 4E, marked by asterisks). Posterior to the middle bands, 3 weak stripes appeared and anteriorly one additional band was detected (Fig. 4E, marked by "o”). During germ band extension (Fig. 4F), a faint set of stripes (Fig. 4F, marked by square) appeared between the 3 posterior bands marked with "o”. Hence, at this stage, a row of 10 distinct bands with different intensities emerged, subdividing the region at 40% to 80% EL of the embryo into a striped pattern, reminiscent of segment polarity gene expression (Fig. 5). During extended germ band (Fig. 4G), all segments exhibited strong Kr expression, mainly in specific neuroblasts (nbl). During germ band retraction (Fig. 4H), strong Kr expression was in neuroblasts of the ventral nerve cord (VNC) and in certain muscle precursor cells (as future). During late embryogenesis (Fig. 4I), Kr was strongly expressed in the brain (B) and the ventral nerve chord (VNC). Kr expression has been analyzed in a variety of insects: Drosophila melanogaster (Jasinski et al., 1986; Gauthier et al., 1988), Musca domestica (Bottinelli and Tuutti, 1991), Culex pipiens (Rohr et al., 1989; Garcia-Bellido et al., 1986), Oncopeltus fasciatus (Wu and Kaufman, 2004). To determine its expression pattern at blastoderm and early gastrulation, resembling that of D. melanogaster (Kr) (Bottinelli and Tuutti, 1991), similar banding patterns were seen in the interior, middle (Fig. 4D) and bifurcated onset of the posterior weak stripes (Fig. 4E, F).

Expression of even-skipped (eve)

Expression of eve began at early nc 14 (Fig. 5A) revealing a broad band with diffuse ends. At early nc 14 (Fig. 5B), an anterior strong band was visible (Fig. 5B, arrow), along with some weak bands (Fig. 5B, snowheads). At mid-nc 14 (Fig. 5C), a more defined banding pattern appeared with broad anterior and posterior bands, respectively. In the middle, a strong unilateral transition band appeared. Thereafter, but still during mid nc 14 (Fig. 5D), the posterior broad band split up, and a new band, band 4, appeared. Anteriorly, the broad band (band 1–2) remained together with the identity of the middle band (referred to as "5") still remained obscure. Close to cellularization (Fig. 5E), all bands resolved, with the exception of band 3 which was still weak. At early gastrulation (Fig. 5F), 7 bands with a width of 3 cells evolved without regular spacing. Notably, band 7 was more posteriorly localized. During germ band extension (Fig. 5G), a new band II appeared which was the only one to remain at extended germ band (Fig. 5H). During germ band retraction (Fig. 5I), a segmental pattern at certain tracheal cells emerged while the posterior band remained defined. At retracted germ band (Fig. 5J–K), the pattern remained unchanged in the tracheal cells, while neuroblasts showed eve transcripts in a repetitive pattern. During late embryogenesis (Fig. 5L), eve transcription remained high in neuroblasts and the posterior spiracles.

To compare the relative position along the A-P axis and the spatial geometry of the eve stripes in some related phylogeny, D. melanogaster and L. erinaceus embryos were stained with monoclonal antibody 256, known to detect the Eve protein in distant insect families. While 256 did not detect Eve in B. donacia (data not shown), it revealed 7 stripes in D. melanogaster (Fig. 5M) and L. erinaceus (Fig. 5N). Interestingly, in comparison to B. donacia (Fig. 5F) where the stripes were perpendicular to the A-P axis, the angles of the anterior-most and posterior-most stripes were tilted towards the dorsal side, respectively (Fig. 5N,M). While this could easily be explained in the case of D. melanogaster by the fact that the dorsal side was rather straight and the ventral side rounded if...
Expression of engrailed (en)

B. dorsalis en expression was first detected at late no. 14 in a two-cell-wide stripe and a posterior weaker stripe (Fig. 5A). The stripes correspond to stripes 3 and 5 and were within the position of the first appearance of the striped en expression of eve (insert in Fig. 5A). Shortly thereafter, at cellularization (Fig. 5B), stripes 9, 11 and 13 emerged, comprising the first wave of en expression in odd-numbered bands. During early gastrula (Fig. 6C), the odd-numbered stripes were complemented by a pair of evenspotted stripes in between, a pattern which progressed through germ band extension (Fig. 6D,E) with 2 cells expressing evenspotted followed by a gap of 4 cells, making a total of 6 cells/segment (insert in Fig. 6E). At extended germ band, even- and odd-numbered stripes reached identical intensities (Fig. 6F). Anteriorly, stripes 0 and posteriorly stripes 15 and 16 became visible, making a total of 17 stripes. The width of the air bands was 2 cells and the interstripe region 8 cells (insert in Fig. 6F). Hence, one segment comprised of about 10 cells at this stage. During germ band retraction (Fig. 6G), a striped pattern in the ectoderm in the posterior segment was visible, which persisted during the remaining stages of embryogenesis (Fig. 6H). The number of en-expressing cells increased to 4 cells and the interstripe region to 12 cells, making a total of 16 cells/segment (insert in Fig. 5H).

Evo-Dervo: comparative expression analysis reveals distinct interpretation of the gap signal at the pair-rule level

A survey in the literature revealed that the expression of Kr as investigated in many insects, a comprehensive list is presented above. Hence, it serves as a prime marker to understand how gene expression correlates with the geometry and layout of the three embryonic. Of these aforementioned insects, only a handful were long germ band insects: Drosophila melanogaster (Gao et al., 1987), Musca domestica (Boettler and Tautz, 1997), Ephemera baltica (Lemke et al., 2010) and L. sericata (Blechert et al., 2011). This collection of data nevertheless permitted the establishment of a map of their expression domains with respect to the A-P axis (Fig. 7A). This comparative map revealed that the majority of long germ band insects showed Kr expressed in a band between 40-53% EL, with L. sericata showing a distinct anterior shift (30-45% EL, Blechert et al., 2011) and B. dorsalis showing a posterior shift (65-80% EL). Surprisingly, when eve banding patterns were compared (Fig. 7B), these considerable differences at the gap-gene level were almost completely restored. The anterior L. sericata eve bands were still more anterior compared to the reference bands of D. melanogaster and B. dorsalis, however, the percentage of the differences compared to Kr diminished. Interestingly, the posterior-most bands were at similar levels, despite the difference of 12-15% at the posterior end of the Kr bands. We conclude that a compensation of the relative position of the segmental anlagen must have occurred during the stage from the gap gene to the pair-rule gene level which is more pronounced at the posterior part of the embryo.

To address the question if gap genes showed a conserved behavior of controlling the pair-rule genes between the three insect families, we superimposed the Kr expression domains to the eve stripes (Fig. 7B). We noted significant differences: In D. melanogaster, the anterior margin of the Kr band is congruent to the posterior margin of eve stripe 2, consistent with the notion that Kr drives the expression of eve stripe 2 (Smillie et al., 1996). In L. sericata, we noted the dramatic anterior shift of the Kr domain (Blechert et al., 2011), but when this domain was superimposed to that of eve, the anterior margin of the Kr band was congruent to the posterior margin of eve stripe 1, and not to that of stripe 2. In B. dorsalis, however, the anterior margin of the Kr band was congruent to the posterior margin of eve stripe 2, as in Drosophila, suggesting a similar control. What was most common to all three insect families was the fact that the Kr band straddled two adjacent eve stripes.

Fig. 7. Schematic representation of relative expression domains of Krüppel and even-skipped in long germ band insects.

(A) Expression domains in regard to the A-P axis in percentage of egg length in D. melanogaster in insect families, as reported (Gao et al., 1987; Roh et al., 1999; Summar and Tautz, 1997; Lemke et al., 2010) along with those of B. dorsalis. Note the anterior anterior shift in L. sericata (Blechert et al., 2011). L. sericata shows an anterior shift in L. sericata (Küpperschmidt et al., 2013) along with those of B. dorsalis. Note the anterior anterior shift in L. sericata (Blechert et al., 2011).

(B) Expression domains of even-skipped (yellow) and spatiotemporal Krüppel (blue) in regard to the A-P axis in percentage of egg length in D. melanogaster (Fig. 5A). L. sericata (Fig. 5N) and B. dorsalis (Fig. 5E). Representative mRNA ISH patterns of embryos are indicated and are shown to scale. The number of nuclei on the dorsal as well as ventral side is indicated. Angles of the bands regarding the A-P axis are indicated in yellow as they appear in the respective insect.

The ratio of the A-P axis is indicated on the right side, followed by the perfect parallelity of the bands in B. dorsalis with respect to the A-P axis, compared to the other insects.
from Epipyrrhus baealisus indicated that the eye stripes were at similar positions as those of Dryopidia (Lemke and Bohrlich-Ott, 2009), but since the K+ band was narrower in comparison to that of Dryopidia (Fig. 7A), it did not encompass the eye stripes as in Dryopidia and consequently, only a single stripe, eye stripe 3 was contained within the K+ domain.

**Evo-Devo: comparative analysis of the shape of the embryos and consequences for the segmentation gene expression at blastoderm stage**

Insect embryos have distinct overall shapes where the primary determinant of the shape is the ratio of the A-P axis versus that of the dorsal-ventral (D-V) axis. To investigate the cause for this, the number of cells for the A-P axes on the dorsal and ventral side were counted in 3 insect systems, D. melanogaster, L. sericata, and B. dorsalis. It should be noted that not only the cell number, but also the size and shape of the individual cells contribute to the overall shape of the insect eggs (Brecher et al., 2011, Meller th et al., 2009). For Dryopidia, on average 106 ± 3 nuclei on the dorsal side versus 104 ± 3 nuclei on the ventral side were counted. For Luocia 120 ± 4 nuclei on the dorsal side versus 131 ± 5 nuclei on the ventral side, and for Bactrocera 126 ± 6 on the dorsal side and 125 ± 3 nuclei on the ventral side (Fig. 7B). The ventral side of the Dryopidia egg was more rounded, despite the fact that both sides have similar numbers of cells. At close examination, this difference is due to the fact that dorsal cells are highly columnar and the amount of lateral cytoplasm is small, enabling a straight dorsal surface (Fig. 7B). Conversely, ventral cells were slightly constricted at the basal side which enabled a rounded surface. The form of the insect embryo is already determined during the late stages of oogenesis, an observation extended to all long germ band insects. Furthermore, the migration of the nuclei to the periphery during noc 9-10 is a morphotropically driven process not involving changes to the shape of the embryo. Hence, the nuclei of the Dryopidia embryo adapted to their environment once they have reached their final position. This difference was also seen in the Luocia egg, however, which also shows a rounded ventral surface, is not as pronounced as in Dryopidia. Due to its elongated shape, it appears to follow another strategy. The number of nuclei on the ventral side is larger than that of the dorsal side, consequently, leading to almost even spacing on both sides. Bactrocera embryos show similar numbers of nuclei on either side and a slightly rounded ventral side which appears to be controlled in a similar way as in Dryopidia.

To address the question whether the different number of cells in the dorsal and ventral side have implications for the expression patterns, a comparison of the angles of the eye stripes with respect to the A-P axis in all 3 insects was conducted. It was noted that the A-P axis of Bactrocera was not always perpendicular to the A-P axes, particularly the first and the last stripes. While this behavior can be explained by the curved nature of the ventral side in Dryopidia (Fig. 5A), a reasonable explanation could not be given for the elongated Luocia egg (Fig. 9). In contrast, a similarly shaped egg such as the Bactrocera egg revealed almost perfectly arranged perpendicular stripes (Fig. 5D-F). Notably, all B. dorsalis segmentation genes analyzed exhibited this behavior at blastoderm stage (Fig. 2-4).

As far as the overall shape of the embryo, there were distinct A-P vs. D-V ratios between the insects. Dryopidia, a relatively compact insect egg showed a ratio of 3.8, while Bactrocera showed a ratio of 3.6 and Luocia as the largest of the 3 eggs had a ratio of 4.3 (Fig. 7). It is important to note that the ratio has an impact on the mechanical stability of the egg; rounder eggs are more pliable than elongated eggs. This fact becomes an issue when the egg is manipulated, e.g., by picking it with a needle during genetic transformation where Luocia and Bactrocera are particularly vulnerable, compared to Dryopidia (unpublished observations).

**Discussion**

We have analyzed the expression of some of the important early A-P axis patterning genes in B. dorsalis and found some similarities to known expression patterns in Dryopidia, but also some distinct features associated with this oviparous fly. Furthermore, we described, in detail, its embryogenesis and imaginal disc development.

We noted some similarities of the appearance of the B. dorsalis egg to that of L. sericata or D. melanogaster. All three insects showed a similar number of segments, including a head, 3 thoracic segments, 8 abdominal segments, and a tail (Fig. 1K). The elongated egg was an excellent marker for segment number as well as identity, hence, comparison of the eye stripe numbers and position might give some clues as to the subdivision of the insect. As evident from Fig. 8, B. dorsalis revealed more anterior stripes than L. sericata (Mellerth et al., 2009) or D. melanogaster (Attil et al., 2016). This is due to a stripe in the head, designated "0" (Fig. 8F) and stripe 16 at the posterior end, while the number and position of the stripes in the trunk seemed constant. These different numbers of stripes may be attributed to different functions of tissues. For example, B. dorsalis females harbor an ovipositor while a true equivalent of this organ is not found in L. sericata or D. melanogaster.

It is important to note that in Dryopidia, not all cells of the cellular blastoderm contribute to the larval larval body structure, referred to as the "late map of the blastoderm" (Scharr and Campos-Ortega, 1998). The process (Fig. 1K) is complex, located at about 35%, E, and AB – at 80%, E, whereas regions more anterior were precursors for head structures and elements of the internal digestive system such as anterior midgut or esophagus. At the molecular level, parasegments were defined as the metameric units to subdivide the blastoderm embryo, whereby the eye stripes define all odonoterminated parasegments (Martínez-Arias and Lawrence, 1985). In this respect, it is noteworthy that in Dryopidia, parasegment 3 which is defined by eye stripe 5 or an stripe 3 corresponds to the anterior half of T1. Hence, neither eye stripe 1 nor en stripes 1 & 2 contribute to the cuticle pattern, but rather to head structures. Likewise, at the posterior end, eye stripes 7 and en stripes 15 constitute AB. When creating a genetic map of the position of the eye stripes among the 3 different insects, it became evident that the number of the lateral cuticle cells were located at distinct regions of the blastoderm (Fig. 7B). In this respect, if the eye stripes serve as a landmark to what extent the blastoderm cells will become precursors for the excisionation of the larva, only about 45% of the surface of the Bactrocera ovariode cells (Fig. 8F) will contribute to the excisionation which is lower than in Dryopidia (95%) or Luocia (53%).

As far as the major tool of segmentation genes was concerned, Bactrocera lacked maternal and which is a major player in this top of the hierarchy of segmentation genes in many insects. It is a
generally accepted view that higher Dipterans, bed bugs through a duplication of an ancestral Holometabola gene. In more basal Diptera where bed bugs is lacking, it was suggested that the function of Jacob is exerted by a maternally - as in zygotically-expressed

Heterochromatin gene (Stauber et al., 2022). In higher Diptera, these functions are now separated into two functions exerted by maternally-expressed and zygotically-expressed enhancer gene (Stauber et al., 2002). In short germ band insects such as Tribolium where Jacob is lacking as well, it was proposed that hox and ast together exert the function of bed (Schröder, 2003).

Very recently, a report in the mitotic Chironomus showed that pax3, a protein containing a cysteine-rich domain (C-terminal motif, can serve as a landmark for development in the Drosophila [Kamp et al., 2015]). Like Jacob, pax3 was strongly expressed at the anterior tip of the tail bud embryo as a result of maternal deposition, and like Jacob formed a maternal gradient at blastoderm stage. Flies-mediated knock-down of pax3 showed revealed a bicoidal phenotype similar to that of Wolbachia. In this stage, pax3 is taken up by the growing Wolbachia, forming a cell. Leptotene stage in the Wolbachia genome. More pax3 is detectable in two closely-related chromosome species which suggested that the anterior expression is actually a very recently (Hübner et al., 2015).

The next class of genes in the hierarchy of the segmentation, the gap genes class show that hox and Kr genes are conserved. Compared to Drosophila where the first expression was observed during nc 15 (Knipling et al., 1986; Bender et al., 1986), B. bavarica showed expression from nc 13 on, suggesting that blastoderm identity followed a distinct regulation. During later embryogenesis, however, identical organs were labeled suggesting that the later functions of both genes were repressed. The first gap gene, gnt, also showed its expression delayed by one nc, compared to Drosophila (Fallenstein and Perrimon, 1993).

Drosophila shows that the pairing rule gene class (Nasokawa-Volhardt and Wieschaus, 1989) where the embryo was analyzed. Compared to Drosophila, the number of even stripes remained conserved. However, it was the evolution of the stripes that was clearly different in the two insects (Macdonald et al., 1995; Fig. 5). Again, transcription of even in B. bavarica was delayed by one nc, compared to Drosophila (Macdonald et al., 1995). In addition, even stripes in B. bavarica were aligned along the A-P axis between the two insects (in B. bavarica, 5F P9 vs. Fig. 3A); these apparent differences at anterior and in Drosophila (McKearin et al., 1987), which is likely due to regulatory differences. The orientation of the stripes must have a different origin.

Our comparative analysis in Fig. 7 demonstrated that there are marked differences across insect families of how the gap genes interpret their pattern on pair-rules genes, evidenced by the position of the anterior boundary in comparison to the even stripes. Even stripes is a paradox of how the maternal input, together with the gap genes, control the pair-rule genes (Silva et al., 1996). While Drosophila shows a similar overlap of the Kr bands with reference to the even stripes where the anterior border of the Kr band was precisely adjacent to the posterior border of even stripe 2 (Fig. 7B), the position of the Llucala Kr band was distinct and was moved exactly one even stripe unit to the anterior. Moreover, even stripe 4 which in Drosophila and B. bavarica is probably activated by Kr (Fig. 7B) does not overlap any longer with the Kr band in Lucilia. Likewise, in Episyrphus balteatus, even stripe 4 does not seem to involve Kr regulation either, as the posterior part of the Kr band does no longer overlap with even stripe 4 (Lemière et al., 2010, Lemière and Schmidt-Ol. 2006). From this comparative analysis, we can conclude that regulation of even stripes 2-4 by Kr is not conserved among insect families. During blastoderm stage at nc 14, we noted a particular behavior for B. dorsalis nuclei, as they did not undergo an elongation step as in L. sericea or D. melanogaster. In Drosophila, elongation of the nuclei is initiated by the formation of an invented base of microtubules that originate from the centrosomes that are located in the periphrasis next to the nuclei (Foe et al., 2000; Foe et al., 1993). These microtubules guide the migration of a tug which migrates from the apical to the basal side of the nuclei, until they wrap the nuclei into a cell. The leading edge of the tornus is driven by an axon microfilament interaction that migrates, from the apical to the basal side, along the microtubules, until the cell membrane encapsulates the nucleus fully, thus forming a cell. The elongated form of the cells at cellular blastoderm allow cell divisions which are one of the driving forces for gastrulation. In contrast, the B. dorsalis nuclei hardly elongated during nc 14 and instead stayed largely round (Fig. 1C). This was true even for gastrulation when the cells are being constricted and for the cellularization that are important for gastrulation. We presume that in B. bavarica, the mechanisms leading to the driving forces for ventral furrow formation or germ band extension must be different ones, compared to D. melanogaster of L. sericea.

During analysis of segmentation genes in L. sericea (McKearin et al., 2006), we noticed that the ratio of the area of nuclei outside to that of the denticle bands varied dramatically when comparing the large L. sericea to that of the small Drosophila. In Lucilia, we measured a ratio of 3:1, while the one in Drosophila was 1:1.6 (McKearin et al., 2006). We also noted that this ratio was particularly dependent on the patterning activity of the segmentation gene list which in Lucilia showed signalling activity over 3 times the distance compared to Drosophila (McKearin et al., 2006). Further, using mathematical calculations, it was shown that a 20-fold increase of the Wingless protein was allowed (McKearin et al., 2006). In the case of the B. bavarica cuticle, we noted that this ratio was 1:2.1 (Fig. 1H) which was closer to that of the small Drosophila, but not the large that of L. sericea. Hence, the ratio of the nucleus to cuticle itself does not increase linearly with the size of the insect. Rather, these ratios likely represent adaptations of the systems to their habitat or their ability to exert movements. We therefore reasoned that these differences in these ratios should immediately be reflected in the expression patterns of the nuclei cuticle-patterning genes of these biological systems. In all cases, the en gene is good marker gene, as it allows to define several areas important for insect segmentation, for the following reasons: (i) its anterior expression border delineates the parasegmental border, a feature which is conserved across most insect phylogeny (McKearin et al., 2006), (ii) the number of expressing cells is a good estimate how large the nuclei cuticle will be (McKearin et al., 2006), (iii)
the number of en bands is a good marker for estimation of how many segments a fly has (Baumgartner et al., 1987; Malleret et al., 2000). When compared to Lucilia where 6 cells were reported to show an expression at extended germband (Meinhardt et al., 2000), B. dorsalis en expression was in much less widespread band, comprising only 2 cells (Fig. 5 F). Hence, the capacity to pattern the cuticle is limited, taken into account the size of the insect. In fact, the much smaller Drosophila embryo also revealed 2 cells expressing en which corroborated the notion that this did not matter.

MATERIALS AND METHODS

Maintenance of B. dorsalis

If dorsalis flies were maintained in 25 x 60 cm round Petri dishes and fed with a constant source providing 3 parts of sugar, 1 part of yeast hydrolysate, and water. For larval stages, a mixture 1000 g per 100 g yeast hydrolysate. 0.5 g yeast extract and 50 g peptone, supplemented by banana or apple pieces was used. To prevent escape of larvae during the “jumping phase” of larval stages, containers were always covered with a cover during this.

Embryo collection

Embryos were exposed to standard food for 10 days. For embryo collection, a smaller plastic beaker was prepared with many small holes (diameter < 1 mm) which was positioned over a freshly-cut apple piece. To make a precise embryo collection, a pre-collection phase of 2 hours was employed where eggs that were deposited were discarded. In some cases, an apple, cut into a halves, was used for embryo collection, where the embryos were collected by removal of the shell and collecting the embryos from the inner surface of the shell.

Identification of orthogroup in B. dorsalis

The Bactrocera orthologues of segmentation genes were identified through standard BLAST searches of a database where access was provided by Scott Bess. This database is accessible via the FBBI Bactrocera oblonga Genome Release 100. Since the maternal braid gene is not present in the Bactrocera genome, B. dorsalis braid RNAi was synthesized and injected into B. dorsalis eggs. Two days later, injected and non-injected larvae were compared for defects in their development.

In situ hybridization

Template preparation, probe preparation and in situ hybridization were performed using 0.4-1 h. B. dorsalis DNA library (Lot. Ngem) packaged, as template and T7 RNA polymerase binding sites on the reverse primer from the above-identified B. dorsalis segmentation genes. These DNA templates were purified, sequenced and used as templates using a DIG-labelling kit (Roche, as described by Fahrni et al., 2014). A non-related sense probe was used as a negative control. In situ hybridization followed a protocol according to (Fahrni et al., 2014).

Immunohistochemistry

D. melanogaster and L. sericata embryos were heat fixed and stained with a monoclonal antibody against the even-skipped protein. (Krogh, 2009; DSHB) at a concentration of 1:250, counterstained with DAPI and monitored on a Zeiss LSM 710 confocal microscope.

Acknowledgements

S. & S. thanks the Swedish Research Council, the Olle Engkvist Byggmästare Foundation the Knut and Alice Wallenberg and the Eric Philip Foundation for support. We thank Kassan Sizemore for access to the B. dorsalis database prior to publishing their data, and Dr. Kari Rehacek for excellent technical assistance and advise for comments on the manuscript.

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