Posterior development
A growing body of evidence

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Introduction

A longstanding question in the field of developmental biology is how embryos specify their embryonic axes, grow from a single fertilized cell to a complex organism and pattern their tissues accordingly to their position. These questions are being increasingly approached from an evolutionary standpoint. On the one hand developmental features have appeared to be evolutionary conserved, on the other hand regulatory strategies in one species are sometimes very different in other species[1]. The mere presence of homologous genes in the genome is no guarantee for homologous functionality. Therefore determining the ancestral state of development can only be inferred by considering a wide variety of species, rather then only the classical model systems, and by considering both their phylogenetic relationship and molecular details of their development[2].

In this review we focus on the process of posterior elongation, which occurs after gastrulation. During gastrulation, a range of events take place, such as invagination, ingression, involution, epiboly, cell intercalation, delamination and extensive folding[3,4], Some of which can be specific for a certain phylogenetic branch, and are not always easily comparable among species. Furthermore, in the first stages of development literature is sometimes incoherent about either assessing cell fate or cell position, and even the definition of axes is not always straightforward, as anterior-posterior, rostral-caudal and oral-aboral are used often for the head-tail axis, but are not per definition equivalent. Several of these problems are also recently reviewed by Stern et al[5]. A further complication is that early axes are sometimes transformed between early and later stages in development.

In any case, the anterior-posterior (head to tail) axis post-gastrulation is thought to be homologous among metazoans, which is reflected in part through strong conservation of Hox genes in a similar anterior to posterior fashion. However, the way this axis is specified in early development can differ significantly. Several different strategies are visible for posterior development in modern day animals, for which only detailed molecular and developmental comparisons can determine whether they are derived, or rather ancestral. The strategy of a posterior growth zone, in which somites are generated progressively in an anterior-posterior fashion, is found in vertebrates. In the classical model system Drosophila, an Ecdysozoan (Figure 1), anterior to posterior develops very differently. Tissues for the entire animal are already laid down in the early embryo, without the need for continuously adding tissue to the posterior after gastrulation. This turns out to be a highly derived mode of development, as other insects and also non-insect athropods posses posterior growth zone, with similarities to vertebrates, indicating that this mode of development was either already present in the the common ancestor of deuterostomes and protostomes[6,7], or at least potentially present. Here we compare the role of essential effectors of posterior development such as the homeodomain Cad/Cdx genes, the Wnt pathway, the FGF pathway, Hox genes and the T-domain transcription factors throughout evolution.

Athropods

Athropods are part of the Ecdysozoans, one of the two branches of Protostomes, the other branch of Protosomes being the Lophotrochozoans. The phylum Athropoda consists of 4 subphyla, hexapoda (including insects), crustaceans, myriapods (including centipedes) and chelicerates (including spiders) (Figure 1).

A recent review on athropod development already highlights the differences in development between Arthropods, and compares their development with that of vertebrates[8]. Within the Athropods we will first focus on insect development. Insects can have many different body lenghts, but generally they have a head region of 6-7 segments, a thoracic region of three segments and an abdomen of 8-11 segments[9]. Although insects in adult life have a similar body plan, they can have very different ways of developing, as also reviewed by Rosenberg et al[9]. Insect developmental strategies are often divided in long-germ-band and short-germ-band development. Although this is the most common division in insect development, insects actually employ a range of different developmental strategies[10]. The long-germ-band insects start development with a large number of presumptive embryonic structures, whereas the short-germ-band insects start development with a small number of presumptive embryonic structures, which are present in the ventral posterior part of the embryo (Figure 2). The latter type of insects generate pattern their body progressively during development, while the long-germ-band insects rather specify and differentiate tissue for the whole axis which is present from the onset of development. We will focus on Drosophila first, as this classical model system has been studied the most extensively.

Long-germ-band development

The basic scheme of development for long germ band developers, is that maternal 'coordinate genes' turn on zygotic transcription of gap genes, which subsequently are responsible in a combinatorial manner for expression of pair rule genes. These pair rule genes are expressed in stripes, defining pairs of segments. Pair rule genes can be subdivided in primary pair rule genes, which are activated by maternal and gap genes, and secondary pair rule genes, which are downstream of the primary pair rule genes. After the segment are formed, their cells receive an anterior and posterior identity due to the 'polarity' genes expressed within the segment (Figure 2). This way all segments are defined at the same time, after which homeotic genes are expressed, responsible for defining the type of tissue to be generated. These homeotic genes are responsible for inducing developmental program of a segment. These homeotic genes have been identified by their ability to induce their respective developmental program ectopically, inducing wings, antennae, legs or halters when expressed in an alternative segment[11]. Although *Drosophila* is the best-studied insect, this type of development is untypical for arthropods and even other insects, as it is a derived mode of development[10].

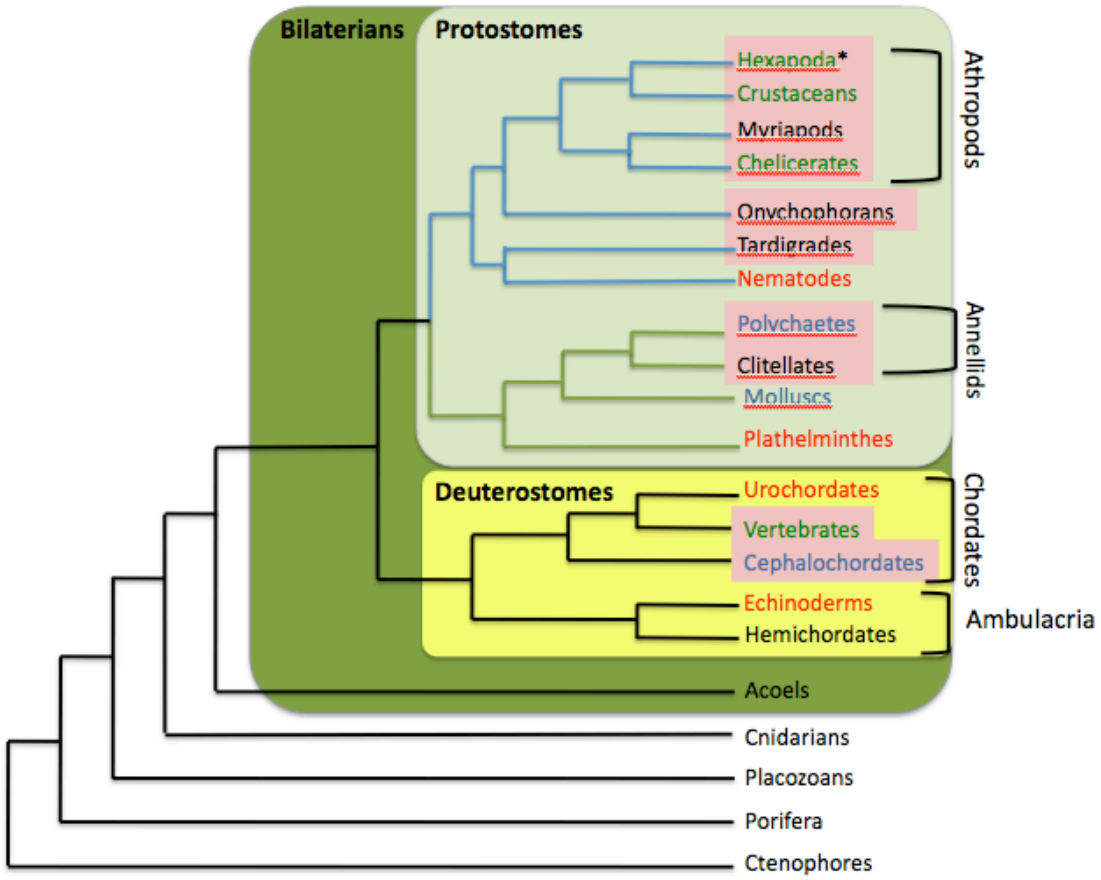

Figure 1. Phylogenetic tree of life, adapted from Damen 2007, Kimelman 2009 and Martindale 2009. The position of subphyla within Ecdysozoans is based upon Dunn 2008 and from Kimelman 2009. Note that Myriapods and Chelicerates are grouped differently then Damen 2007, based upon Dunn 2008. There is controversy about both the position of Placozoans as well as the Ctenophores, see also Philippe 2009 and Telford 2009.
Blue branch = Ecdysozoa, Green branch = Lophotrochozoa; Pink box = Segmented phylum, red letters = no growth zone, green letters = Growth zone controlled with Wnt and/or Caudal, black letters = possible growth zone, but no expression data, blue letters = Growth zone with Wnt/Caudal, but no functional experiments
*Hexapoda is made up of insects and entognaths. Most insects develop with a growth zone, however not exclusively.

In *Drosophila* maternal mRNA's are already localized to specific regions of the egg cell during oogenesis. An example in the anterior region is *bicoid*, while in the posterior region maternal *nanos* mRNA is localized. It should be noted that the prominent role of *bicoid* is an example of the derived mode of development for *Drosophila*, as *bicoid* is a gene found specifically in a subgroup of insects, namely the *Diptera*, the flies[8]. However the neighboring gene in *Drosophila*, *Zerknullt,* is present in other species. Both *bicoid* and *zerknullt* are thought to be homologues of the *Hox3* gene, which is much more

conserved throughout bilaterians[12]. This raises the possibility that *bicoid* has taken over specific functions, which in other arthropods is fulfilled by *Zerknullt*, or other homologous genes. There are many other (maternal) mRNA's distributed throughout the embryo, of which many are only translated locally. This is the case for *caudal* and *hunchback* which are only active in the posterior and anterior respectively, and form protein gradients across the embryo[13].

At this point, let us also introduce a second long germ band insect, the wasp *Nasonia*. In *Nasonia* the gene *otd* is responsible for anterior patterning, and is distributed in a gradient from the anterior instead of *bicoid*[14]. However, *Nasonia* also expresses *otd* at the posterior, a notable difference with *Drosophila*[14]. Regardless of this difference, both species require the gene *caudal* for posterior development, a gene encoding a homeodomain containing transcription factor. However, whereas *Drosophila* expresses *caudal* mRNA throughout the embryo, and only translates it at the posterior, *Nasonia* already localizes the *caudal* mRNA to the posterior, creating an mRNA gradient[15]. Functionally both strategies lead to a protein gradient, with the highest concentration at the posterior end. This illustrates how crucial developmental genes, can be subject to different regulatory strategies, even in closely related species.

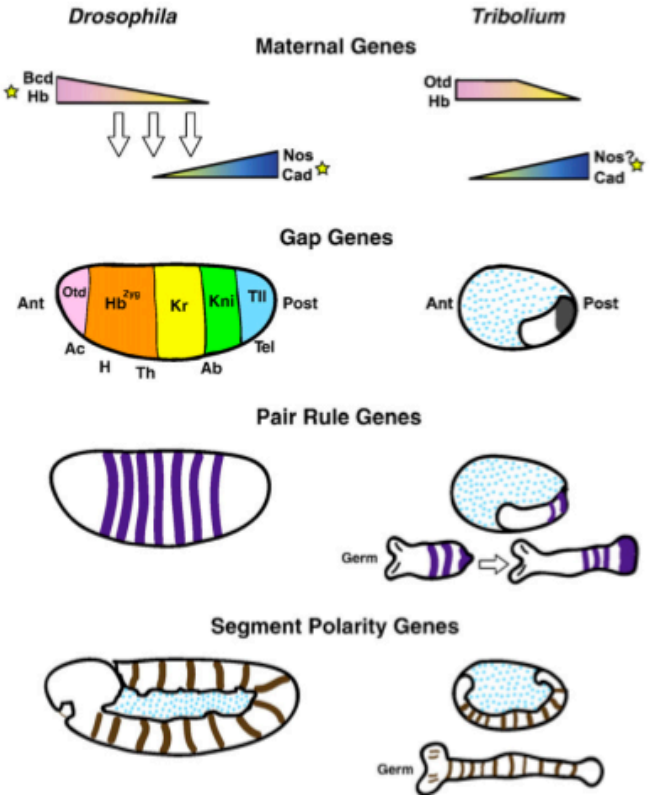

Figure 2. Adapted from Rosenberg et al. 2009[8]. Typical developmental strategy of long-germ insects and short-germ insects. Both species use gradients (depicted by triangles) with a maximum on either the anterior or posterior side, after which gene expression domains are estabilished. Note the simultaneous patterning in Drosophila, versus the gradual development in Tribolium.
Ac = acron, H = head, Th = Thorax, Ab = abdomen, Tel = telson.

Short-germ-band development

The prevalent form of development found within

Arthropods is short-germ-band development. Arthropods employing this type of development, have progenitors for the anterior part of the embryo only at the early embryonic stages, and develop a posterior growth zone after gastrulation from which cells and tissues are continuously generated in a cellularized environment (Figure 2). There are several short germ band developers that are emerging as model systems, such as the flour beetle *Tribolium*, but also several types of spiders and crustaceans.

After a process reminiscent of convergent extension found in vertebrates, the germ band is extended ventrally to the anterior. After the head is already formed, segments are continuously added to the posterior, due to the presence of a 'growth zone'[16].

Cad

In *Artemia* (crustacean, which develops with a growth zone), *Tribolium* (short-germ band insect), *Schistocerca gregaria* (grasshopper, a short germ band developer) and *Gryllus bimaculatus* (a cricket, and intermediate-germ band insect) *caudal* is expressed in the posterior growth zone[17,16,18]. In *Tribolium* expression starts broad, but becomes restricted as soon as posterior development starts, while in *Artemia*, expression is already restricted to the growth zone from the start of early development[16,19]. For *Gryllus* the expression of *cad* is more similar to *Tribolium*, with a broad initial expression, after which it is restricted and becomes localized to the growth zone. In *Schistocerca* the situation is slightly different, as during early development specialized cells along the membrane of the egg express *caudal* briefly, after which *caudal* expression is restricted to the most posterior part of the embryo. When *cad* knocked down by RNAi, severely truncated animals develop for all four of these species, showing that *cad* is functionally similar and essential for axis elongation (Supplementary table 1). Knockdown of *cad* inhibits formation of segments, and also disrupts normal gene expression, such as *engrailed* and *even-skipped*, indicating an upstream role for caudal in axis elongation and patterning[16]. This is different from *Drosophila*, where *cad* is responsible for the most posterior part of the embryo[20], but is also shown to regulate expression of the pair rule gene *ftz*[21].

The centipede *Strigamia*, a myriapod, also expresses a caudal homologue during formation of its posterior. Interestingly, *cad* displays waves of expression, rather than static expression, so that stripes of caudal expression move anteriorly from the growth zone, together with transcripts of other pair rule genes[22]. It seems that in these species caudal is not restricted in developing the posterior structures, but rather is incorporated in segmentation. Although this is quite a different role than in vertebrates, (discussed later) the waves of expression are reminiscent of a 'clock and gradient' model, proposed to underlie somitogenesis in vertebrates[23-25]. Indeed the question of whether or not there is a segmentation clock in insects has also been posed before[26].

Wnt

In *Tribolium* canonical Wnt signaling plays a role in axis elongation as well as posterior patterning, as loss of Wnt signal leads to truncated animals as well as segmentation

defects, especially when both Wnt8 and Wnt1 are knocked down or downstream Wnt targets such as *pan* (TCF in vertebrates) or *porcupine* are depleted[27]. In *Gryllus* knockdown of Wnt/Arm is also responsible for loss of *cad* expression in the posterior growth zone in an early stage, and leads to a truncated animal, similar to *cad* knockdown[28]. However, while in *Drosophila cad* only seems to be functional in the posterior, in *Gryllus* there are also defects in more anterior segments. However this makes sense, considering the fact that for this type of progressive patterning *cad* is also required in earlier stages, when the more anterior segments are laid down. Unfortunately *cad* has not (yet) been shown to be a direct target of *wnt* signaling, which would identify similarity in gene hierarchy between arthropod and vertebrate cad/wnt interaction.

In a chelicerate, the common housespider *Achaearanea*, *Wnt8* is responsible for posterior growth, and its loss by pRNAi leads to loss of posterior segments and expression loss of several downstream targets such as *caudal* and *Notch*/*Delta*[23]. Although *Wnt3* is present in the genome of even very 'basal' animals such as cnidarians, no *Wnt3* gene has been isolated from protostomes, indicating a branch specific gene loss.

Hox

The developmental gene cascade, as described for *Drosophila* anterior-posterior development, is conserved the strongest at the level of segment polarity genes and pair-rule genes, while there seems to be less conservation in the upstream regulation among athropods[8]. Especially between *Drosophila* and *Tribolium* there are some interesting shifts in hierarchy, for instance in the pair rule genes[29]. In *Tribolium* the secondary pair rule gene *odd* functions as a primary pair rule gene, while the gene *hairy* does not function as a primary pair rule gene, as it does in *Drosophila*. Regardless of some species specific changes, it seems likely that the pair rule genes already played a prominent role in the arthropod ancestor[30]. Besides the differences in hierarchy, because of the differences in developmental strategies between *Drosophila* and *Tribolium*, loss of function of pair rule genes show different phenotypes. By knocking down primary pair rule genes in Tribolium, which are expressed in the growth zone, the embryo develops into a truncated animal[29]. This shows that pair rules genes in *Tribolium*, are not just involved in setting up boundaries but also on the process of axis elongation.

T/Bra/Byn

The gene brachyury is a T-domain transcription factor and was discovered as one of the first genes in vertebrates to be required for posterior development, and is also present in arthropods. The homologous gene *brachyenteron* is expressed in the growth zone as well as in the hindgut of *Tribolium*. However, it seems to have no role in axis elongation in *Tribolium*, when knocked down using RNAi, as the entire animal is formed[31]. RNAi directed against *byn* efficiently inhibits hindgut formation, without any form of truncation. However since this study did not confirm the levels of knockdown, it could be possible that growth zone specific *byn* was still present. In an intermediate germ band developer, the cricket *Gryllus Bimaculatus*, knockdown of Byn/Bra leads to the

same phenotyope, loss of the hindgut, but shows no defects in segmentation or axis elongation[32]. Additionally it was shown that knockdown of byn/bra had no effect on either *wnt* or *cad* expression in the posterior growth zone, but only disrupted *wnt* and *cad* expression in the intestines and hindgut. In Drosophila, in which a growth zone is absent, a similar role is observed for the *byn/bra* gene in the hindgut. Upon creation of six different point mutations, loss of *byn* function leads to defects development of the hindgut and anal pads, similar to short germ developers[33]. Interestingly, loss of *byn* leads to aberrant initial expression of *cad*, which is subsequently lost early in development compared to wild type flies.

This represents a notable difference with the situation in vertebrates, where Brachyury is required for AP tissue formation, and is also expressed in nacent mesoderm and plays a role in notochord development, discussed below.

Torso

One type of genes not yet discussed are the 'terminal genes' which are known from Drosophila, to be expressed in both the acron and telson, the most terminal parts of the embryo. One example is *torso* and *torso-like* (*tsl*), which in Drosophila specify both the most anterior and posterior part (although in some conditions is not required for head formation[34]), which also plays a role in Tribolium. Parental RNAi (pRNAi) knockdown of these genes, leads to loss of posterior segments[35]. Knocking down *torso* or *tsl* also prevents expression of *cad* and *wg* (wingless). In *Drosophila* Wnt expression is also under control of torso[36]. As parental RNAi knockout of *torso* in *Tribolium* results in the development of normal head and anterior segments, but lacking all structures posteriorly which normally develop, this suggests that the processes developing the anterior germ and subsequently developing the posterior are two separate processes[35]. Although *torso* and *tsl* are required for normal development in several insects[37], no homologues have been identified in other arthropods or more distantly related animals, indicating this is a highly derived feature of development.

Vertebrates

Several vertebrate model systems are used to study development, such as the chicken, frog, zebrafish and the mouse. All vertebrates share the developmental similarity of progressively generating tissue, including somites in an anterior to posterior (rostral-caudal, head to tail) fashion, and have fascinated developmental biologists for decades. More recently the molecular basis for this process has been integrated, defining a molecular clock, and molecular gradients involved in developing the posterior, which has also been reviewed extensively[23,38-40,24]. Briefly, a posterior gradient of FGF and Wnt is present, which are at a maximum in the growth zone and decline towards the anterior. (Figure 3) An opposing gradient of retinoic acid helps is required to define the 'determination front'. Besides these gradients there are waves of gene expression. These emanate from the posterior, concerning the Notch, Wnt and FGF pathways[25]. It is striking that the genes in tissue generation also play a crucial role in somitogenesis.

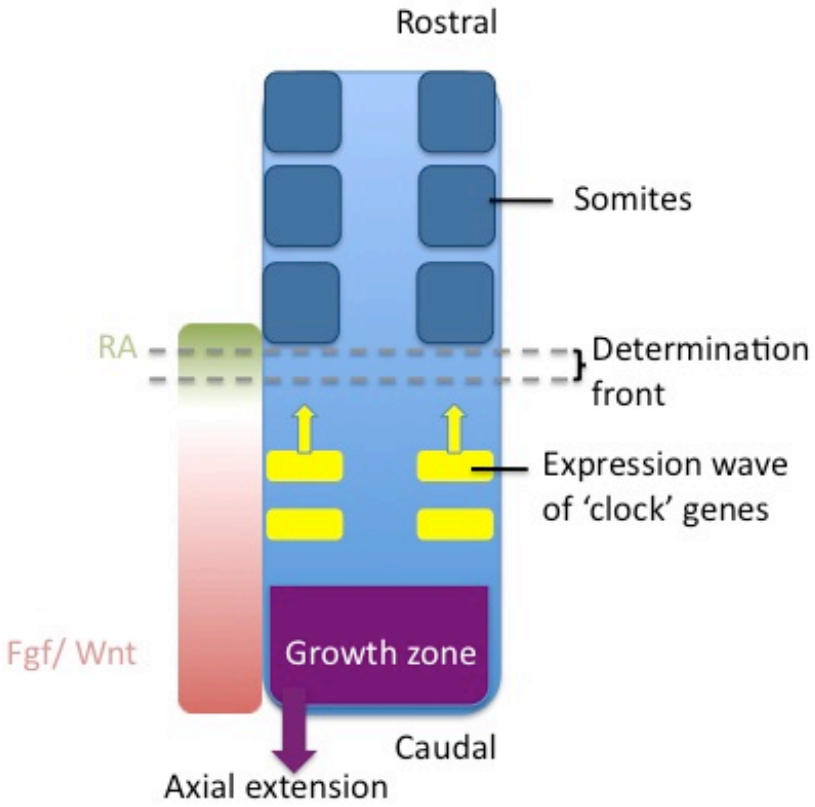

Figure 3. A simplified model of axial extension and somite differentiation based on several reviews, Holley 2002, Aulehla 2004, Dequéant 2006, Dequéant 2008. An posterior FGF/Wnt gradient and anterior RA gradient are essential for posterior tissue generation and maintain a determination front, where presomitic mesoderm (PSM) differentiates to somites. Waves of expression of cyclic genes originate from the growth zone, moving rostrally. In the determination front they induce somite formation in competent cells in the correct environment. Axial extension also migrates the Wnt/FGF gradient relieving cells from a high concentration as the growth zone moves posteriorly.

Zebrafish

Cad/Cdx

The homeodomain containing transcription factors that are known as cad or caudal in insects are named *Cdx* (Caudal related hox) in vertebrates. Zebrafish posses three Cdx genes, Cdx1a, Cdx1b and Cdx4. Cdx4 was identified to be important for posterior body formation, as well as formation of blood progenitors[41]. Cdx4 is required for expression for a set of Hox genes, which in turn are required for correct tissue differentiation. Cdx1b rather seems to be involved in formation of the endoderm and differentiation of the intestines[42]. The Cdx genes in zebrafish seem to be downstream of both FGF and Wnt signaling, which makes them suitable candidates for inducing somite formation in the posterior.

Both Cdx1a and Cdx4 expression are reduced by inhibition of Wnt3a/Wnt8 combined morpholino injection, or by canonical Wnt inhibition by injection of Dkk. Interestingly these Cdx functions are specific for posterior body formation, as other Wnt mediated functions, such as head formation or regulating the organizer remained unaffected[43]. As mentioned, Cdx1a and Cdx4 are under control of FGF signaling as inhibition by a specific FGF inhibitor, SU5402, or injection of a Xenopus dominant negative form of FGF8 reduces expression of Cdx1a and Cdx4. Conversely, injecting FGF8 leads to misexpression as well as expanded expression of both genes[43]. These results do not rule out that FGF and Wnt signaling independently regulate Cdx expression. However in the Wnt3a/Wnt8 morpholino injected embryo's there was no change in the downstream FGF target *sprouty4*, which indicates FGF signaling was not perturbed, and the Wnt observed tail defects are FGF independent[44].

Wnt

Wnt is also one of the three major pathways in the hypothesized 'tail organizer' in zebrafish, which is hypothesized to be a different signaling centre than the 'trunk organizer'[45]. This tail organizer utilizes Wnt, BMP and the Nodal signaling. All three are shown to be required for normal tail formation. As both Nodal and Wnt8 have an auto and cross stimulatory effect as well as both stimulating BMP2B, the combination of either Nodal and Bmp2B or Wnt8 and BMP2B is sufficient to stimulate tail formation[45]. Interestingly overexpression of these three pathways is even able to produce multiple ectopic tails.

The interplay between Wnt and BMP signaling is not quite clear, as injection of specifically Wnt8 leads to induction of BMP2B[45], while a different study reports that only a combined knockdown of Wnt3 and Wnt8 leads to loss of BMP2B expression in lateral ventral tissue, but not in the tailbud[43]. Similar discrepancies currently surround Wnt and Nodal signaling, as Wnt8 is found to increase znr1 and znr2 (zebrafish nodal related) expression[45], but knockdown of Wnt3/Wnt8 seems to have no effect on *Cyclops* (a Nodal homologue), or rather increase than inhibit expression[43].

Since Wnt signaling is closely involved with other signaling pathways, it is hard to identify direct targets and effects. However one of the direct target genes of Wnt signaling is sp5l, a transcription factor containing multiple zincfinger DNA binding domains. When inhibited by morpholino injection, it shows only a mild tail defect, however in combination with Wnt3a knockout, it shows a strong increase of tail developmental defects. Additionally, sp5l RNA injection can also rescue Wnt3a morpholino defects, showing it is a direct downstream target of Wnt signaling[44].

Other downstream targets of canonical Wnt signaling are Ntl and Bra, (discussed later), as a heat shock inducible dominant negative form of TCF completely abolishes all Ntl and Bra expression[46]. Conversely, Ntl/Bra loss of function embryos show normal Wnt8 expression in early developmental stages, but show significant loss of expression after gastrulation, indicating a specific role of Ntl/Bra in maintaining Wnt signaling during posterior development. Moreover, Ntl/Bra overexpression also induces upregulation of Wnt signaling[46].

T/Bra

In zebrafish several T-box transcription factor genes involved in posterior development have been identified, most prominently *no tail* (Ntl), *spade tail* (Spt) and *Brachyury* (Bra). Within this class the first homologue of the mouse *brachyury* was *ntl*[47,48], which leads to formation of only 17-19 of the 32 somites when mutated to a non-functional protein. *Spadetail* does not seem to be required for posterior growth, but rather for correct cell movement and mesoderm development, as mutants lack several somites and form a 'spade' formed tail filled with mesenchymal cells which seem to be unable to migrate anteriorly[49,50]. Recently another homologue of *brachyury* was identified, named *Bra*. *Bra* is actually more similar to the mouse brachyury homologue than Ntl. As mutations in mouse brachyury lead to a more severe phenotype in mouse than was observed in fish in *no tail* mutants, functional redundancy of *no tail* and *Bra* was the suspected cause. Unexpectedly, when inhibiting *Bra* with morpholino injections, no phenotype is observed. However when Bra is inhibited in a *Ntl+/-* or *Ntl-/-* background, only 8-12 somites develop, indicating that *Ntl* indeed has a redundant function for *Bra*, and that both homologues play a role in posterior elongation and somitogenesis.
A series of transplantation experiments helped determine whether Bra/Ntl function in a cell autonomous or non autonomous fashion. Donor cells from Bra/Ntl morphants as well as from Ntl-/- mutants were able to form somites when transplanted in wildtype developing zebrafish. Conversely also cells from wildtype fish were transplanted to Ntl/Bra morphants, which led to rescue of somites, and partial increase of expression of downstream targets of Ntl and Bra, indicating that not only the donor cells contributed to improved axial extension, but also neighboring cells responded to the donor cells. Therefore it is clear that *Ntl* and *Bra* function in a non cell autonomous manner[46]. It should be noted that Bra and Ntl function in a cell autonomous way for notochord formation, indicating different signaling pathways, in specific tissues[47,46].

FGF

The FGF protein family has 22 members in vertebrates, which bind to 4 specific FGF receptors[51]. These receptors contain two or three extracellular immunoglobulin like binding domains, and a heparin binding domain. Intracellularly, tyrosine kinase domains further transduce the signaling pathway, after extracellular ligand binding. Inhibition of multiple or all FGF's by the FGF inhibitor SU5402, leads to pregastrulation defects[52]. By inhibiting a general eFGF (embryonic FGF) dominant negative receptor, defects in gastrulation as well as truncated phenotypes were detected, depending on the injected amount of mRNA[53]. By creating and expressing different dominant negative FGF receptors (dn-FGF), the role of FGF signaling could be examined in more detail, although the specific ligand is not immediately clear[54]. All zebrafish expressing dn-FGFr developed a truncated posterior, however dn-FGFr3 showed the most severe phenotype. A soluble form of this dn-FGFr3 protein (sdn-FGFr3), showed to efficiently inhibit FGF8, suggesting that FGF8 is the crucial extracellular FGF is posterior patterning. Post gastrulation FGF8 is expressed in a gradient, high in the tailbud and declining towards the anterior, although it is also expressed in specific regions in the head[55]. FGF8 mutant zebrafish are unable to complete gastrulation, emphasizing an important role in early development. Recently the nature of this gradient was also shown on a protein level, which is described by a 'source-sink' model, in which the proteins can diffuse freely[56]. Interestingly, while in Xenopus a positive feedbackloop exists between FGF and Brachyury signaling[57,58], in zebrafish this is only true for the axial mesoderm, which will form the notochord. In the paraxial mesoderm, which will form the somites, loss of Ntl/Bra does not lead to a reduction of expression of FGF targets[46]. The specific target gene investigated is *pea3*, a downstream target of FGF8 specifically[59]. As mentioned before, FGF and Cdx are found to cross regulate each other[43], while it is not yet clear whether FGF is under direct control of Wnt, as in mice[60]. Interestingly the gradient formed by FGF8 influences somite size: by inhibiting it's posterior signal larger somites are formed, while transplanting FGF8 beads anterior of the developing somites leads to smaller somite formation[61].

Considering the above leads to postulation of the following model. Note specifically that there is no regulation of Wnt by FGF, or at least not when considering FGF downstream target *sprouty4* as readout[44].

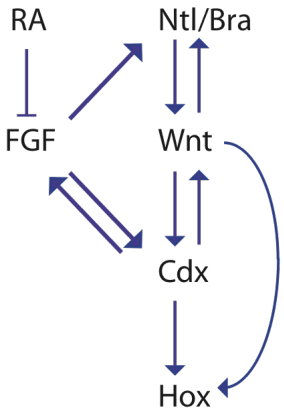

Figure 4. Model of posterior development in Zebrafish. RA, FGF and Wnt are present in a gradient in the posterior. RA forms a rostrocaudal gradient, while FGF and Wnt are present in a caudalrostral gradients. Although not specifically denoted, due to redundance, the most crucial Wnt proteins are Wnt3a and Wnt8. In the Cdx class the isoforms Cdx1 and Cdx4 are the most essential. The responsible initiator of this network remains to be determined, but is likely to activate Wnt.

Mouse

Especially in the mouse there has been a lot of focus on developing and validating different models concerning posterior growth and segmentation.
Now it has become clear that axial elongation requires progenitor cells under the influence of molecular gradients, and that tissue patterning and growth is also regulated by these gradients as well as by a molecular clock.

both a gradient and a molecular clock are responsible for correct posterior growth[23]. Many separate genes have been identified to play a role in posterior development (Supplemental table 1 and elswhere[39,40]), of which functional inhibition leads to posterior truncation. In recent years not only the role but also the interplay with the other genes are becoming elucidated.

Cad/Cdx

Mice have three paralogues of the Cad/Cdx homeodomain transcription factors, Cdx1, Cdx2 and Cdx4. Especially for Cdx1 and Cdx2 it is clear that they are involved in posterior development, thanks to the creation of compound mutants. Cdx2 plays the most pronounced role in axis elongation of the Cdx genes, and also gives the most severe phenotype (Supplemental Table1). Cdx4 rather seems to play a role in correct development of the allantois, and placentogenesis in a redundant manner with Cdx2[23].
While Cdx1-/- mutants only show mild posterior defects, Cdx2-/- mutants are not viable, and several heterozygous mutants with intermediate phenotypes show that these two genes function redundantly in elongating the A-P axis. Cdx2+/- mutants display an anteriorization of lower cervical and thoracic vertebrae, while Cdx1+/- Cdx2+/- mutants loses even more caudal vertebrae, and the most severe posterior truncation is found in Cdx1-/- Cdx2+/- mutants, showing the synergistic function of these genes[62]. A similar increase in phenotype is found for Cdx2+/- Cdx4 -/- compound mutants, which leads to truncation as well as vertebrae fusion. By a knock in model where Cdx1 was replaced by Cdx2 by homologous recombination[63], the redundant function of these genes was further confirmed, consistent with the above described mutant studies.

How Cdx genes are responsible for posterior development in mice is not completely clear but several reports show that Cdx genes regulate expression of at least several Hox genes at specific timepoints[64-66]. Cdx transcription factors act by binding to *cis* regulatory elements, which have also been found in Hox genes[65]. It is unclear whether Cdx genes posses a homeotic (confers spatial information about the type of tissue should be formed), as they clearly have in Drosophila[20], or rather have an instructive role, regulating downstream targets, such as Hox genes[67].

However direct rescue of Cdx phenotypes by Hox was recently published, without upregulating Cdx transcription, showing that Hox genes can play a role in controlling

both generation of tissue as well as instructing it[68]. In this study Hoxb8 and Hoxa5 rescue the axis-impaired phenotype in Cdx2 +/- Cdx4 -/- (Cdx2/4) mutants. These Cdx2/4 mutants were also rescued by expressing a target gene of canonical Wnt signaling Lef1, showing that Cdx and Hox are likely upstream of Wnt signaling[68].

Neither Cdx1 nor Cdx2 are likely to directly affect T/Brachyury expression, as the expression is still present in the mutant mice, albeit restricted to a smaller region, as the posterior region is reduced[62].

It should be noted that Cdx genes are not only required for posterior elongation but are also involved in development and patterning of the mesoderm and the neuroectoderm[62,69].

Wnt

Wnt signaling is pivotal in posterior development of the mouse, integrating the 'segmentation clock' and the FGF gradient required for normal development[23]. Especially Wnt3a and Wnt5a are required for axis elongation and correct posterior development[70,71]. Mutating Wnt3a to a nonfunctional protein is embryonic lethal, and results in an embryo with normal anterior, but truncated posterior. Somites are deformed or absent, indicating a defect in axis elongation[70]. Furthermore, Wnt3a is also responsible, probably indirectly, for the expression of Notch and Delta in a cyclic manner[72]. Additionally Wnt3a is also required for normal levels of FGF8 expression[60], further linking several signaling pathways in posterior development. The most recent hypotheses integrate these three pathways in a cyclic manner, where Notch and Fgf oscillate in phase and Wnt oscillates in a complementary phase[25,24].

As in zebrafish, identifying downstream targets of Wnt signaling is problematic. However there are reports of Wnt3a regulating Cdx1 expression, for which the TCF/Lef binding site are required[73]. The defects observed in Wnt3a mutants were explained by loss of Cdx1, resulting in loss of, or altered, Hox expression[74]. Cdx genes have already been hypothesized to be the bridge between different signals and Hox genes[75]. Cdx4 is also thought to be directly regulated by Wnt3a via TCF/Lef response elements in it's promoter region[76].

T/Bra

The T-domain transcription factor T/Brachyury was already identified in mouse to play a role in posterior body development over 8 decades ago[77], but only since its cloning[78], did the molecular and genetic mechanisms become clear. In homozygote mutant mice posterior truncations are present, which resemble the phenotype found in Wnt3a null mutants[79,80]. Indeed, Brachyury is a direct target of Wnt signaling[81]. A later study confirmed that regulation of Brachyury by Wnt3a is mediated by Lef1, a downstream target of Wnt signaling[82]. Activated Lef1 protein driven by a Brachyury promoter, was able to rescue the Wnt3a homozygous hypomorph vestigial tail mutants, indicating Lef1

truly acts downstream of Wnt3a in posterior development. Furthermore, it is clear that Lef1 is not required for initiation of expression of Brachyury, but rather for maintenance of the signaling[82].

Although Brachyury is critical for correct posterior development, its target genes are starting to be identified only recently. The role of T-box genes during development, focused on early mesoderm differentiation has been reviewed[83], and might show involvement of common pathways with axis elongation. It is also possible that there is cross talk between Brachyury and Cdx genes.

FGF

As in zebrafish, both FGF8 mRNA as well as protein is present in a gradient from tailbud to anterior[84,85]. It seems the mRNA gradient is created by active transcription in the most posterior part, while mRNA decay is responsible for the decline of the gradient. A mathematical model to describe this FGF8 gradient was more recently published[86]. The essential role of FGF8 pregastrulation in zebrafish is conserved, as FGF8-/- mice fail to proceed through gastrulation correctly[87]. Also conserved is the role of FGF8 in determining the boundary of somites, or in other words regulating the size of the developing somites[85]. Similar to zebrafish, increasing local concentration of FGF8 results in smaller somites, while inhibiting FGF8 leads to larger somites. Interestingly the cross regulation between FGF and RA is also conserved. RA is present in a gradient which is in the opposite direction as FGF, thus in an anterior to posterior fashion. Whereas RA inhibits FGF and promotes differentiation, FGF is (indirectly) responsible for degrading RA and thereby keeps the posterior undifferentiated[39,40].

The above data is combined in the following model (Figure 5).

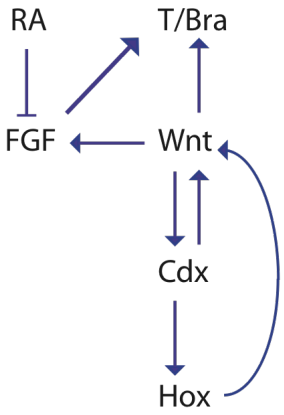

Figure 5. Model for posterior development in the mouse. A high degree of conservation in posterior development is found when comparing zebrafish and mice. The T/Brachyury, Wnt, Cdx, Hox and FGF genes play similar roles. However whether are crossregulations are conserved remains uncertain, for instance so far in mice Cdx has not been shown to be under regulation of FGF. As in zebrafish, the initiation event is likely to regulate Wnt.

Other model organisms

Besides the above described model systems, several species which have not been studied in such detail yet, already provide some interesting data about conservation of genes and their functions.

Amphioxus is a Cephalochordate, the most basal group of Chordates, making it a sister group to the vertebrate and urochordate lineage[88]. It is clearly segmented and develops with a growth zone[89]. In this growth zone, Wnt1, Wnt3, Wnt5, Wnt6, Cdx and Brachyury are expressed, but due to lack of functional experiments, it remains difficult to compare functional regulation with vertebrates. Interestingly, in *Amphioxus* somites bud off from the tailbud itself, rather than from the presomitic mesoderm[90]. Even so *Amphioxus* seems to use a similar Notch/Delta based clock mechanism[90], and retinoic acid and a Wnt gradient are also present[91]. This would indicate that the common ancestor of chordates probably already used a similar clock and gradient for posterior development.

Within Urochordates, which are not segmented, the Ascidian *Halocynthia roretzi*, there is no structure such as the 'tailbud' as found in zebrafish. Nevertheless it also expresses a Cdx homologue in the posterior, which is required for axis elongation[92]. Regulation of Hox genes by Cdx also is conserved as morpholino knockdown of Cdx leads to reduced expression of Hox1. This shows that even in the unsegmented Urochordates a similar network for posterior development is present as in segmented chordates.

The expression of a Brachyury homologue has also been detected in the growth zone of *Patella Vulgata*, a mollusk found within the Lophotrochozoans[93]. It is expressed in the posterior of the body from an early developmental stage, and could indicate that the role of *brachyury* in the posterior is conserved, possibly for axis elongation or maintaining a stem cell pool. If it turns out to be required for axial elongation it would suggest that posterior elongation is the ancestral role of Bra in bilaterians, which would be secondarily lost in Athropods, or possibly only in Ecdysozoa, although tardigrades and onychophorans will need to be studied to provide a more definitive answer about the ancestral state of both the last common ancestor of protostomes and deuterostomes.

Even in the sponge (Porifera, figure 1) *Amphimedon Queenslandica* Wnt is present and expressed at the posterior pole of the larvae[94], indicating that these genes are ancient. Even though there is some evidence that the function of Wnt signaling is also present in sponges[95], only three paralogues are present without orthology to other metazoan Wnt genes. Therefore it is seems unlikely that Wnt signaling will show a conserved gene network between the sponge and bilaterians, in which Wnt is further duplicated and recruited in posterior development.

Stem cells and progenitors in posterior development

Posterior development with a growth zone, either in arthropod or in vertebrate, requires cells that self renew as well as differentiate, defining them as progenitors or stem cells. Where these cells are located and how they are kept in an undifferentiated state are open questions. Two types of experiments that have helped investigate this question are grafting experiments with labeled cells and retrospective clonal analysis, reviewed Wilson et al[39].

Currently the most complete picture of axial elongation and somitogenesis, which has been elucidated at the molecular level, is found in vertebrates. By different studies in mouse, chick, frog and zebrafish it has become clear that Wnt, Notch, BMP, FGF and retinoic acid all play a pivotal role in axis extension[39,40,24]. The spatio-temporal regulation of posterior development is summarized in models with a 'segmentation clock' and 'wave front' (Figure 3)[23-25]. Here we will not focus on somitogenesis, which is a patterning event, but rather on the regulation of stem cells and progenitor cells in the growth zone.

From series of transplantation experiments in mice it is clear that progenitor cells can behave as stem cells after transplantation and change their gene expression and fate of their descendants. This shows that cells in the tail bud are not only autonomously regulated, but environmental cues greatly influence their development[96].

In zebrafish, Brachyury is suspected to play a role in keeping progenitors undifferentiated[46], although it remains unclear how. One potential regulator is Wnt3a. Wnt3a transfected mouse embryonic stemcells significantly upregulate Brachyury in culture[97]. In similar cell culture experiments Wnt1, 3a and 4 specifically upregulated Brachyury, which is dependent on a TCF/LEF-1 binding site, situated upstream in the promoter region. Importantly, Brachyury is also a downstream target of Fgf, which has also been suggested to keep progenitor cells undifferentiated. Fgf8 keeps cells which would normally form somites in an undifferentiated state with a mesenchymal character. These cells also retain expression of Brachyury, similar to cells in the growth zone[85].
The opposite retinoic acid gradient is thought to be responsible for cellular differentiation. Therefore genes encoding RA degrading enzymes such as Cyp26a1, are also responsible for keeping the growth zone undifferentiated. Cyp26a1 is under control of Cdx genes in both mice[68] and fish[98], linking Wnt signaling to RA clearance.

Conclusion & Discussion

Model systems

Throughout evolution the molecular and cellular mechanisms of axial growth seem to have been at least partially conserved during evolution. Although this review covers species of both the Protostomes as well as Deuterostomes, by now it is clear that a strong focus on a single, or only few organisms, can lead to erroneous hypothesis about their evolutionary relationship. Also for this review not all animal models have been dealt with. For sake of readability and space, we omitted species such as sea urchin (an echinoderm), Xenopus, chick and C.Elegans to name but a few. However future research will likely include these and others, as several phyla are severely under represented. Within Protostomes the Onychophorans and Tardigrades are only starting to be sequenced[99,100]. Both phyla are segmented, however information about genes involved in posterior growth is not yet present (Figure 1)[7]. Unraveling their genetic regulatory network should prove helpful in determining the Protostome ancestral state. But also within the Deuterostomes there are gaps to be filled. While the vertebrates are the most extensively studied, the Cephalochordate Amphioxus has also attracted interest as model system[89], as well as the Hemichordate Ptychodera Flava, an acorn worm, which is likely to be sequenced soon[101].
Since gene networks are more likely to persist through evolution than a single gene, researchers face the difficulty of having to analyze a network, instead of an individual gene. With the rise of new sequencing technology and an interdisciplinary approach between the Evo-Devo field and Systems Biology, more definitive answers as new exciting questions can be expected.

This has put researchers in the situation, where it clear that both more species, as well as more genes and their interactions have to be studied, to make sensible inferences about ancestral gene networks.

Models of gene networks

Regardless of the species, it has become clear that during embryonic development, many different signaling pathways are employed to orchestrate axial growth from anterior to posterior, as to finely regulate differentiation, as well as ensure robustness. Although some of the components are ancient, their ancestral roles are hard to discern. It is clear that both Wnt and brachyury are ancient, as they are also present in cnidarians[102,103]. However Brachyury does not seem to play a similar role in posterior development in species outside of Deuterostomes. In all Ecdysozoa studied so far, *byn* is expressed in the posterior, but is not required for axis elongation, but rather differentiation of the hindgut (Supplemental Table 1). The ancestral role of Brachyury is clouded further by the fact that several lineage specific gene duplications have occurred. Therefore, even within Deuterostomes, it is likely that there are species-

specific novelties in regulation. These networks are also rapidly complexifying as new interactions are identified. Whereas previously models mostly suggested a signaling pathway in which Wnt was upstream of Cdx genes, which in turn regulate Hox genes[73,75], also upregulates Wnt, establishing a positive feedbackloop[68].

Homology in posterior development?

When trying to determine the mode of posterior development of common ancestors of phyla, and the likely gene regulatory network, we are limited to the phyla to which are current model systems belong too. For Protostomes, our current knowledge is most extensive in the Athropods, in particular from insects. For Deuterostomes, the vertebrates are the best studied phylum.

Both in Athropods and Vertebrates the role of Caudal/Cdx genes and Wnt signaling seems deeply concerned with posterior development. However while Wnt3a is the central pillar for vertebrates, this gene has likely been lost in Protostomes. However by knocking down canonical Wnt downstream targets, Protostome embryos develop similar posterior truncation as vertebrates. The role of Wnt is also found conserved between Protostomes and Deuterostomes, illustrated by Wnt8 in the zebrafish and the spider. Furthermore also the homeodomain containing transcription factor Cad/Cdx is present in both phyla, and is expressed in the growth zone. In both species the Cad/Cdx and Wnt interactions seem conserved, even though it seems that in Protostomes Cad genes might already fulfill a role in differentiating the tissue rather regulating downstream genes such as Hox genes.

Currently, the most interesting similarities suggesting common origin in posterior development come from the beetle[27] and the spider[104]. Especially in the spider it seems that there are waves of expression originating from the growth zone, similar to vertebrates. However, whether or not gradients such as Fgf, Wnt and an opposing RA gradient have not yet been identified in Protostomes. Since maternal mRNA's have been shown to be present several insects, it would not be surprising if zygotically transcribed genes would also lead to gradient formation.

Segmentation issues

If posterior development through a growth zone does turn out to be homologous, and therefore present in the common ancestor of Protostomes and Deuterostomes, does this mean that the this ancestor was also segmented? It would make it likely, however segmentation and a growth zone are not necessarily coupled. Drosophila shows a strong segmentation without a growth zone, while there are mollusks which develop with a growth zone, but shows no overt segmentation[7]. However the growth zone of mollusks has hardly been studied, and might turn out be highly derived. Even so, another argument indicating that segmentation between Protostomes and Deuterostomes has arisen independently, is that Athropod segmentation is coordinated by the ectoderm, while in vertebrates this is driven by mesoderm[105]. As for axial elongation, the only way

to determine whether or not segmentation is ancestral or derived, is by increasing the number of phyla under study and approaching these questions in an interdisciplinary manner, combining Evo-Devo with systems biology.

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