Review article:

A comprehensive review of the molecular development of zebrafish (*Danio rerio*)

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Abstract

Despite the extensive use of zebrafish (*Danio rerio*) as a model organism, a unified source detailing its molecular development remains lacking, particularly regarding germ layer formation and axis patterning. This review provides a comprehensive overview of the molecular mechanisms underlying zebrafish development. Following fertilization, the zygotic genome remains transcriptionally inactive, and maternally deposited factors determine the precise positioning of zygotically active signaling centers that guide body axis formation and patterning. The dorsal and ventral signaling centers antagonize one another to establish the dorsal–ventral (D/V) axis. Their interaction gives rise to inductive morphogen fields composed of Bmps, Wnts, Fgf, and Squint, which generate concentration gradients that specify distinct mesendodermal and ectodermal cell fates along the D/V axis, ultimately shaping the body plan (fate map). Along the animal–vegetal axis, a decreasing gradient of Squint toward the animal pole contributes to the sequential specification of endoderm, mesoderm, and ectoderm. According to this model, high levels of Squint promote endoderm formation, lower levels drive mesodermal development, and its absence permits ectoderm specification.

Keywords: Zebrafish, Dorsal organizer, Ventral center, Axis patterning

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Introduction

Despite the extensive use of zebrafish, *Danio rerio*, as a model organism, there is no unified source detailing its molecular development, especially regarding germ layer formation and axis patterning. Hence, this review work aimed to provide a comprehensive overview of the molecular mechanisms underlying zebrafish development.

Shortly after the oocyte fertilization, its cytoplasm segregates from the yolk by an outward cytoplasmic streaming called lifting, thereby generating the first blastodermal cell. Then, a multicellular blastoderm cap on top of the large yolk cell will be built by the rapid and repetitious meroblastic cleavages (Kodjabachian *et al*., 1999; Gore and Sampath, 2002). Because the zygotic genome is transcriptionally quiescent after fertilization, maternally expressed factors direct the early embryonic development until the zygotic genome activation (ZGA). In fact, at about 1000-cell stage (3 hours post fertilization (hpf)), the embryo enters a critical point, namely mid blastula transition (MBT) or maternal to zygotic transition (MZT), wherein the zygotic genome transcription and maternal transcripts degradation begin (Schier, 2001; Gong and Korzh, 2004; Leichsenring *et al*., 2013; Voronina and Pshennikova, 2016). In other words, maternal factors trigger ZGA at the MZT when the developmental control transfers to the embryonic nucleus. This universal transition represents a major reprogramming event that requires (Lee *et al*., 2013; Leichsenring *et al*., 2013; Pauklin and Vallier, 2015; Perez-Camps *et al*., 2016): (A) Chromatin remodeling to provide the transcriptional competency, (B) specific activation of a new transcriptional program, and (C) clearance of the previous transcriptional program and maternal mRNAs, which is partially regulated by the conserved miRNA430 (Zhiyuan and Vladimir, 2004; Gong and Korzh, 2004; Leichsenring *et al*., 2013; Lee *et al*., 2013).

RT-PCR and in situ hybridization (ISH) results implied that the pluripotency markers encompassing Oct4, Nanog, and SoxB1 (including Sox2 and Sox19b) are ubiquitously expressed in the zygote. Translation levels of all maternal mRNAs, using the ribosome-profiling data, revealed that Nanog, SoxB1 family, and Oct4 are the most translated transcription factors before the MZT. These factors initiate more than 74% transcription of the genes at the MZT (Lee *et al*., 2013; Leichsenring *et al*., 2013). Zebrafish undergo a very narrow period of stemness condition from the ZGA to a brief moment after the oblong stage (Lee *et al*., 2013). Principally due to changes in their levels and consequently their binding partners, the pluripotency factors function shift from the stemness maintenance (merely expression of the genes involved in the stemness sustaining) to the differentiation induction (expression of the specific markers of the germ layers) (Hasanpour *et al*., 2020). Although this is an exciting area of research, it is beyond the scope of this review (Fig. 1).

Molecular process of development

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Body axes and germ layers formation and expression of their specific markers depend on the zygotically active signaling centers, namely, organizers. Organizers include the Nieuwkoop center counterparts (the dorsal/Spemann-Mangold organizer and yolk syncytium layer (YSL)) and the ventral center. Maternally expressed factors determine the precise location of the aforementioned zygotically active signaling centers (Kodjabachian *et al*., 1999; Ulrich and Heisenberg, 2004; Tuazon and Mullins, 2015; Thisse and Thisse, 2015).

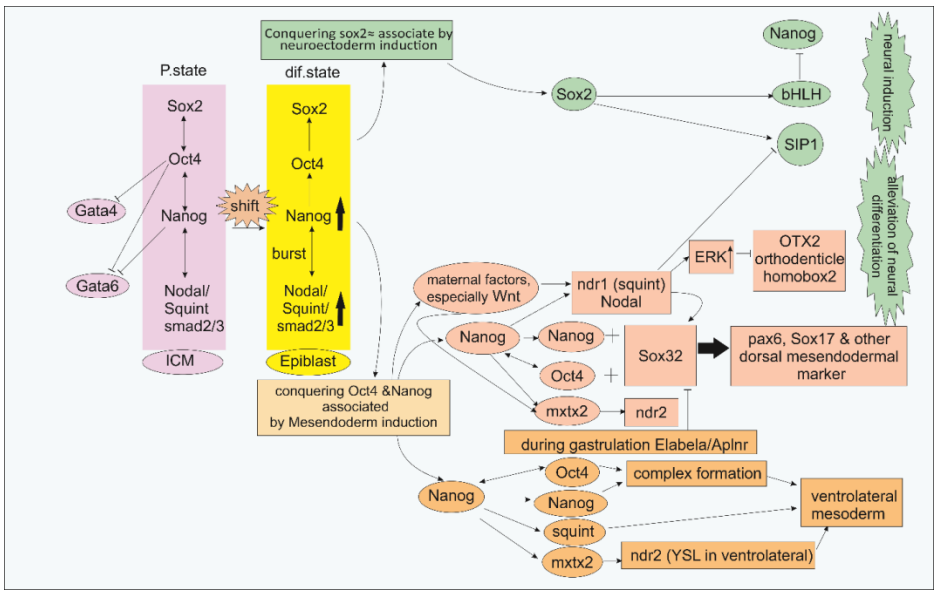

Figure 1: The role of pluripotency factors in germ layer formation in zebrafish.

Principally, the embryo is a battlefield between the ventral and dorsal signaling centers that antagonize each other. In this regard, the ventral center induces the ventral and posterior identities by secreting the Bmps and Wnts, while the dorsal organizer promotes the dorsal structures through the Squint, Chordin, and other factors (Neave *et al*., 1997; Mullins, 1998; Schier, 2001; De Robertis, 2009; Thisse and Thisse, 2015; Tuazon and Mullins, 2015). Additionally, it provokes the anterior fates through the antagonists of Wnts, Bmps, Fgfs, and Squint (Fig. 2). The result of the battle between the dorsal and ventral signaling centers is the creation of such inductive fields of morphogens encompassing Bmps, Wnts, Fgf, and Squint (Liang and Rubinstein, 2003; Schier, 2005; Poulain *et al*., 2006; Tuazon and Mullins, 2015). These conductive concentration gradients contribute to germ layers formation along the animal/vegetal axis and patterning of the different mesendodermal and ectodermal fates along the Dorsal/Ventral (D/V) axis; i.e., the body plan or fate map is formed (Feldman *et al*., 1998; Schier, 2001; Dougan *et al*., 2003).

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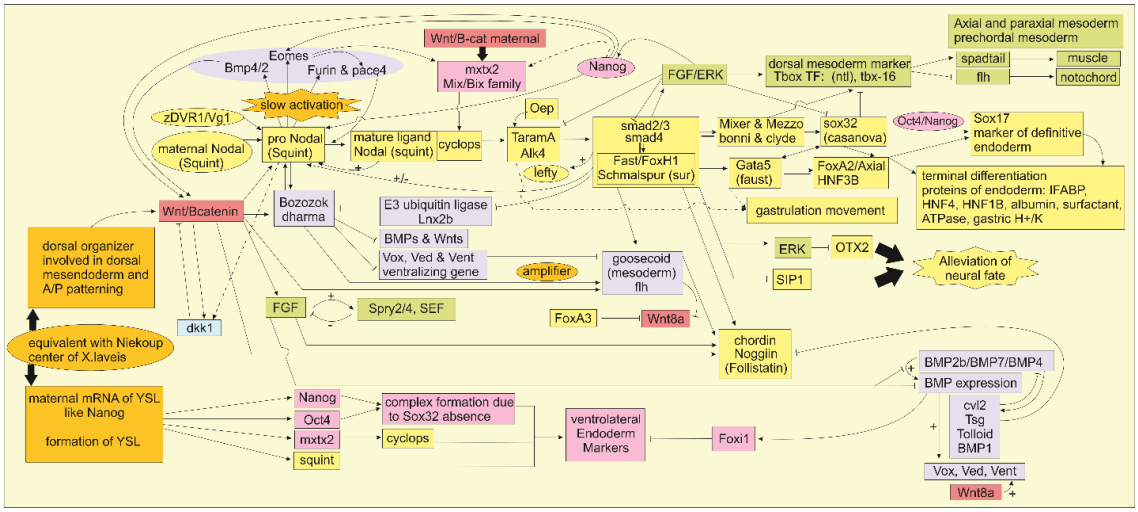

Figure 2: YSL and dorsal organizer involvement in D/V patterning of the germ layers.

Dorsal spemann-mangold organizer

Dorsal spemann-mangold organizer formation and its function

Upon the oocyte fertilization and before the first cleavage, maternal dorsal determinant factors (for example Ichabod, β-catenin, Fgf, zDVR1 and Squint related transcripts), assembled in the vegetal pole of the zygote, are translocated to the future dorsal side of the embryo, through a microtubule dependent process like the cortical rotation in *Xenopus laevis* (Feldman *et al*., 1998; Mullins, 1998; Whitman, 2001; Schier, 2001). Indeed, the assemblage of the dorsal determinant factors in the dorsal side of the embryo leads to the formation of the dorsal organizer and, consequently, the expansion of the dorsal mesendoderm and anterior neuroectoderm. Therefore, either ablation of the vegetal third of the yolk or microtubule disruption by the nocodazol provokes total ventralization (lack of the dorsal mesoderm, neuroectoderm, and the most anterior 14-15 somites) (Mullins, 1998; Schier, 2001; Gore and Sampath, 2002).

As mentioned above, the cortical rotation results in the asymmetric accumulation of β-catenin (*ctnnb1* gene symbol) in the dorsal YSL nuclei and dorsal marginal blastomers. Probably Ichabod and some other unknown factors trigger Wnt pathway activation, ligand independently (Feldman *et al*., 1998; Munoz-Sanjuan and H-Brivanlou, 2001; Gong and Korzh, 2004; Ulrich and Heisenberg, 2004). Following Wnt pathway activation, β-catenin is translocated to the nucleus, where, in concert with the Tcf/Lef family, it induces expression of the zygotic genes that are responsible for turning on the organizer genetic programs. These zygotic factors are as follows: 1- an organizer-specific homeodomain transcription factor Bozozok/Dharma (orthologue with siamus and twin in *X. laevis*), 2- Chordin, 3- Dickkopf1 (dkk1), 4- Fgf, 5- Squint, and probably 6- Goosecoid. Furthermore, the organizer involves in the development by activation of the non-canonical Wnt and retinoic acid pathways too (Feldman *et al*., 1998; Schier and Talbot, 2003;

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Zhiyuan and Vladimir, 2004; Thisse and Thisse, 2015; Tuazon and Mullins, 2015).

Introduction of the zygotic genes induced by β-cat

Bozozok/dharma: The master of dorsalization

Bozozok/Dharma transcribes some dorsalizing factors, including 1- Dkk1, a Wnt antagonist, 2- Squint, 3- Chordin, a Bmp antagonist, 4- Noggin. In addition, its presence is a key amplifier for Goosecoid expression. Furthermore, Bozozok can also act as a transcriptional repressor and directly suppresses transcription of some ventralizing factors, such as Vox (*vega1*), Ved (*ved*), Vent (*vega2*), Bmp2b, and Wnt8a. Taken together, Bozozok directly and indirectly promotes dorsal development. Principally, the Bozozok null mutants lose their axial mesendoderm, such as the notochord and prechordal plate. They are defective in the anterior neural specification based on morphological examination and the lack of forebrain markers, such as Sek1 and Emk1. Additionally, they exhibit cyclopia and fail to express the dorsal determinant factors, including Chordin and Dkk1. Nevertheless, these mutants possess both the Anterior/Posterior (A/P) and D/V axes, suggesting that other factors induced by β-cat, like Fgf or such unidentified ones, manage parts of the key organizer activities too (Kodjabachian *et al*., 1999; Ulrich and Heisenberg, 2004; De Robertis, 2009; Ramel and Hill, 2013; Thisse and Thisse, 2015; Tuazon and Mullins, 2015).

Fgf

Zygotic Fgf is first transcribed at the early blastula by the maternal β-cat. Symmetry breaking of the Bmp gradient is caused by some factors, including Fgf, at around the 30% epiboly. Fgf acts in a dorsal to ventral graded fashion to knock down the Bmps and suppress their positive auto-regulatory loop by promoting the degradation of Smad1/5 as the signal transducers of the BMPs cascade (Ulrich and Heisenberg, 2004; Mizoguchi *et al*., 2006). In addition, at the posttranslational level, Fgf signaling directly represses the Bmps activity by inducing the Chordin expression. Together, the D/V gradient of Fgf8 at the early gastrula dorsalizes the embryos by repressing the BMPs. Furthermore, Fgf activity is attenuated and controlled by an antagonistic feedback loop involving Fgf itself, Sprouty (spry2/4), and Sef (interleukin 17 receptor D) (Mullins, 1998; De Robertis, 2009; Bökel and Brand, 2013; Thisse and Thisse, 2015; Tuazon and Mullins, 2015).

Goosecoid

Goosecoid, a homeodomain transcription factor, activates transcription of the Bmp inhibitors including Chordin, Noggin and Follistatin. Furthermore, Goosecoid and FoxA3 repress *wnt8a* encoding (Schier, 2001; Dougan *et al*., 2003; Thisse and Thisse, 2015; Tuazon and Mullins, 2015). Goosecoid is known as the direct target of Squint because its overexpression can lead to the lone

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transcription of Goosecoid; Bozozok, on the other hand, indirectly promotes Goosecoid expression through the amplification of Squint signaling. In addition, Lnx-2b, an E3-ubiquitin ligase, naturally suppresses the Bozozok transcription and progressively restricts its domain to the dorsal mesendoderm or organizer region, and only a high dose of Squint is sufficient to suppress Lnx2b and subsequently preserve Bozozok. In other words, Bozozok function necessarily requires Lnx2b suppression, which is done by Squint signaling. Taken together, the cooperate function of Squint and Bozozok seems to be critical for the correct spatio-temporal expression pattern of the Goosecoid (Liang and Rubinstein, 2003; Schier and Talbot, 2003; Ulrich and Heisenberg, 2004; Ro and Dawid, 2010).

Chordin

Chordin (encoded by the *short gastrulation gene* (*sog*), formerly known as *chordino* (*dino*) gene) relieves Bmp binding to their receptors through its four cysteine-rich domains (CR) harboring Bmps. Chordin is only expressed in the dorsal marginal zone, but because of its -ange diffusion ability, it expands ventrolaterally and occludes the Bmps. Furthermore, Chordin function is modulated by some other factors such as Twisted gastrulation (Tsg), Tolloid, Secreted Frizzeled Related Proteins (s-FRPs), and Cross vein less two (Cvl2) (Mullins, 1998; Kodjabachian *et al*., 1999; Kondo, 2007; De Robertis, 2009; Tuazon and Mullins, 2015).

The Chordin modulators are as follows: (a) Twisted gastrulation (Tsg) expressed ventrally keeps Bmps in a soluble active state and promotes their signaling. In the absence of Tolloid, Tsg, as a cofactor and modulator of the Chordin activity, mediates a ternary complex formation by binding to both the Chordin and Bmps, thereby inhibiting the Bmps signaling. Although at the same time, Tsg reinforces the cleavage of Chordin by Tolloid as well (Kondo, 2007). (b) Tolloid (*minifin* gene), a type of metalloproteinase produced in the ventral lip, promotes the Bmps signaling and subsequently ventralization by cleaving the Chordin at two specific sites. (c) Secreted Frizzeled Related Proteins (s-FRPs), a class of secreted proteins, Functions as the antagonist of Wnt signaling. s-FRPs containing the homologous domains to the Wnt binding site of the frizzeled receptors bind with the Wnts, and prevent frizzeled receptor activation. However, one of the s-FRPs, namely Sizzled (ogon/mercedes gene), acts as a competitive inhibitor of Tolloid, and inhibits the degradation of Chordin by Tolloid and progressively ventralization. This factor is expressed in response to high levels of the Bmps signaling (Ulrich and Heisenberg, 2004; Kondo, 2007). (d) Cross vein less two (Cvl2) is produced ventrally in response to high levels of the Bmps. Cvl2 is anchored by the glycosylated proteins (Glypicans) to the surface of the cells in which it has been synthesized. It has an antagonistic effect on the Bmps due to its cysteine rich (CR) domains, which enable it to

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bind to the Bmps directly. This ability of Cvl2 is enhanced by Tsg (Kondo, 2007; De Robertis, 2009).

Dickkopf (dkk1)

Dickkopf, a family of membrane bound proteins, is expressed in dorsal domain of the embryo. Dkk1 prevents the propagation of Wnt signaling by blocking its co-receptors, including Lrp5/6 (De Robertis, 2009; Tuazon and Mullins, 2015).

Squint

In *X. laevis,* except the maternal β-cat, vegetally localized maternal transcripts of VegT and Activin-like factors (including Vg1, Activin, and Squint orthologues) cooperatively conduct the early zygotic expression of Squint orthologues. Maternal transcripts of VegT orthologue, encoded by the *spadtail* locus in zebrafish, were not found in the zygote, and loss of the *spadtail* function did not display the same range and severity of defects seen in the VegT null mutants in *X. laevis*. Hence, the VegT functional analogue has not yet been identified in zebrafish. However, the Vg1 orthologue, namely zDVR-1, in zebrafish has been recognized, and its presence as a maternal mRNA has been confirmed. Zygotic Squint expression in the dorsal side of the embryo is triggered by β-cat, Nanog, maternal transcripts of Squint itself, z-DVR1, and zygotic Goosecoid (Ulrich and Heisenberg, 2004; Ho *et al*., 2006; Thisse and Thisse, 2015).

Squint signaling transcribes Squint itself, Bozozok, Dkk1, Chordin, Goosecoid (in coordination with Bozozok), and Fgf (Feldman *et al*., 1998; Gore and Sampath, 2002; Ulrich and Heisenberg, 2004). Squint emanating from the dorsal organizer creates a D/V concentration gradient which involves in the dorsal specification and A/P axis patterning of the organizer in coordination with its Animal/Vegetal (A/V) gradient (it will be discussed later) (Feldman *et al*., 1998; Pogoda *et al*., 2000; Dougan *et al*., 2003; Xu *et al*., 2008; Xu *et al*., 2012; Tuazon and Mullins, 2015). Eventually, Squint signaling after activation of the differentiation programs becomes transcriptionally silent through polycomb-mediated trimethylation of Histone 3 at Lys27 (H3K27me3) (Quail *et al*., 2013).

Ventral signaling center or Bmp4 sync-expression group and its ventralizing factors

The ventral signaling center, previously known as the Bmp4 sync-expression group, battles with the dorsal organizer by employing some ventralizing factors. BMPs and Wnts, two sets of morphogens originating from the ventral center, induce the ventral and posterior identities in embryos (Kondo, 2007; Tuazon and Mullins, 2015).

Bmps family

Bmp morphogens belonging to the Tgfβ superfamily pattern the tissues along the D/V axis. The most prominent Bmps involved in this process are the Bmp2 (bmp2a, bmp2b), Bmp4, and Bmp7. Bmps are expressed uniformly in the

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blastoderm cap. Subsequently, they are gradually excluded from the blastula organizer to the ventral half of the embryo, where Bmps are essential for specifying ventral fates (Schier, 2001; Zhiyuan and Vladimir, 2004; Kondo, 2007; De Robertis, 2009; Tuazon and Mullins, 2015). In fact, except for the higher expression rate of the Bmps at the ventral domain than the dorsal one, the Bmps activity is regulated primarily by their antagonistic factors, expressed at the dorsal side of the embryo (Kondo, 2007; Tuazon and Mullins, 2015).

Bmp4

In contrast, the maternal transcripts of Bmp4, which accumulate in the dorsal blastopore lip, do not play a role in dorsal patterning based on functional analysis. In accordance with its zygotic up-regulation, Bmp4 is expressed ubiquitously at the onset of the blastula stage. However, Bmp4 expression withdraws to the edge of the neural plate because the dorsalizing agents (such as Chordin, Noggin, and Follistatin) conquered it. The Bmp antagonists completely alleviate the Bmp4 expression and function in the dorsal lip, and confine it to the ventral side of the embryo at the gastrulation (Munoz-Sanjuan and H-Brivanlou, 2001; Kondo, 2007; Ramel and Hill, 2013).

Bmp2b (swirl gene) and Bmp7 (snail house)

Bmp2b is expressed in the ventral progenitor and dorsal organizer region (the embryonic shield) at 4hpf (the sphere stage) (While Bmp2a has not yet been recognized by in-situ hybridization). In accordance with the gastrula initiation, the dorsal organizer factors partially refine the Bmp2b gradient by clearing it from the dorsal-most region of the embryo. The graded Bmp activity induces different cell fates along the D/V axis. In addition, the Bmp7 expression initiation is observed in the ventral half of the embryo at the shield stage (Kodjabachian *et al*., 1999; Ulrich and Heisenberg, 2004; Kondo, 2007; De Robertis, 2009).

Admp

Anti-dorsalizing morphogenetic protein (Admp), belonging to Bmps, unlike the rest of its family members, is expressed exclusively in the future dorsal side of the embryo. Its primary expression pattern is restricted to all three germ layers to the axial mesoderm alone. In contrast with its dorsal expression, it amplifies the ventral fate and trunk formation (Kondo, 2007; De Robertis, 2009). Admp expression is initiated by Wnt/β-cat, but Bozozok positively influences its maintenance to reduce the head and anterior structures. Therefore, Admp, acting in concert with BMPs, attenuates the organizer activities in the dorsal side of the embryo. Interestingly, Admp is merely suppressed by Follistatin, and it does not act using the Bmp canonical signaling pathway (Ulrich and Heisenberg, 2004; Kondo, 2007).

Wnts (Wnt11: siber blick (slb); Wnt5: pipetail (ppt)

Maternal Wnt signaling triggers expression of a waterfall of genes

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responsible for the dorsal organizer formation at the early and mid-blastula stages. However, Wnt signaling is excluded from the dorsal organizer at the late blastula to gastrula stage, and then confined to the ventrolateral margin. Wnt signaling pathway participating in the dorsal fate specification is referred to as the canonical Wnt pathway or β-cat dependent. In contrast, Wnt transduction cascade inducing ventralization partly follows a β-cat independent mechanism, regulated by Ca^{2+} and Ca^{2+}-calmodulin-dependent kinase-II (CaKMII). Indeed, both β-cat-dependent and independent mechanisms cooperatively operate in the establishment of the ventrolateral fates. Another hypothesis explains that β-cat probably binds to some different transcription factors to induce the ventral fate (Ulrich and Heisenberg, 2004; Tuazon and Mullins, 2015).

Both zygotic Bmps and Wnts provoke the ventral cell fate through Vox/Ved and Vent expression. Additionally, Wnts and Bmps synergistically promote ventralization because Wnts suppress Gsk3, thereby alleviating its effect on the linker region of the Smad1/5/8, i.e., Wnts promote Smad stability and subsequently Bmps signaling duration (Ulrich and Heisenberg, 2004; Kondo, 2007; De Robertis, 2009; Tuazon and Mullins, 2015). Bmps signaling can partially compensate for the function of Wnt8 at the ventral margin. In this regard, Bmps overexpression rescues the deficient Wnt8 embryos and retrieves the expression of the ventro-posterior mesodermal markers such as tbx6. Together, Wnts in concert with the Bmps are crucial to the formation of the ventrolateral mesoderm (Wu and Hill, 2009).

Vox, Ved, and Vent

These transcription repressors limit the expression territory of the dorsal-specific genes (for example, Chordin, Frzb, and Shh) to the dorsal half of the embryos. Therefore, it is conceivable that they indirectly promote the ventral fate (Schier, 2001; Thisse and Thisse, 2015).

Patterning of the germ layers along the D/V axis; result of the signaling centers' battle

Maternal transcripts of the Bmps accumulated in the dorsal blastopore lip trigger Wnt expression. In fact, immediately after MBT, Bmps are ubiquitously expressed in the embryo; therefore, all maternal dorsal determinant factors like β-cat, which are assembled in the future dorsal side, battle against the most powerful ventralizing factors Bmps, and another one, Wnts (Kodjabachian *et al*., 1999; Schier, 2001; Gong and Korzh, 2004; Ulrich and Heisenberg, 2004). So, until the end of gastrulation Bmps expression gradually limits to the ventral side of the embryo, which is known as the ventral center. The most important factors participating in the gradient formation of the Bmps are the Bmp antagonists secreted by the dorsal-Spemann organizer. Briefly, Chordin, Noggin, and Follistatin are the antagonists of the Bmps at the post-translationasl level. In

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addition, FGF represses the Bmps transcription. Total concerted actions of these molecules set up a ventral to dorsal gradient of Bmps (Mullins, 1998; Thisse *et al*., 2000b; Munoz-Sanjuan and H-Brivanlou, 2001; De Robertis, 2009).

Maternal Wnt signaling establishes the dorsal organizer; however, Wnt signaling is also excluded from the dorsal side due to Wnt antagonists actions produced by the dorsal organizer. Some Wnt antagonists like Dkk1 and secreted forms of the frizzled receptors have been recognized in the dorsal organizer (Mullins, 1998; De Robertis, 2009; Tuazon and Mullins, 2015). Taken together, the result of the battle between the dorsal and ventral signaling centers is the creation of the inductive fields of morphogens encompassing the Bmps, Wnts, Fgf8, and Activin-like factors. These conductive concentration gradients contribute to the patterning of the different mesendodermal and ectodermal fates along the D/V axis, i.e., the body plan or fate map is formed (Feldman *et al*., 1998; Gore and Sampath, 2002; Schier and Talbot, 2003; Schier, 2005; Tuazon and Mullins, 2015). For instance, the Bmps high activity specifies the ventral fates, including the epidermis, pronephros, blood, and tail fin. The intermediate level of the Bmps elicits lateral tissues formation, like the neural crest. Furthermore, no Bmp Signaling results in the dorsal fate (the neural plates and somites) (Schier, 2001; Munoz-Sanjuan and H-Brivanlou, 2001; Tuazon and Mullins, 2015).

The ectodermal cells overlying the organizer and those located in a more ventral position give rise to the neural tissues and epidermis, respectively. Subsequently, the neural ectoderm becomes regionalized along the A/P axis and allocates into the forebrain, midbrain, hindbrain, and trunk neural tissue. Blood is classically defined as the ventral-most mesoderm, whereas the Kidney (pronephros), muscle, and notochord are respectively located in the ventral to dorsal direction in the mesoderm germ layer (Fig. 3) (Munoz-Sanjuan and H-Brivanlou, 2001; Ober *et al*., 2003; Chan *et al*., 2009). Principally, the position of a cell along the D/V axis before the gastrulation reflects its future location with respect to the A/P axis. According to the detailed fate mapping studies at the late blastula stage, the position of the endodermal progenitors in the marginal zone resembles the topographic arrangement of the presumptive digestive system (Munoz-Sanjuan and H-Brivanlou, 2001; Ober *et al*., 2003).

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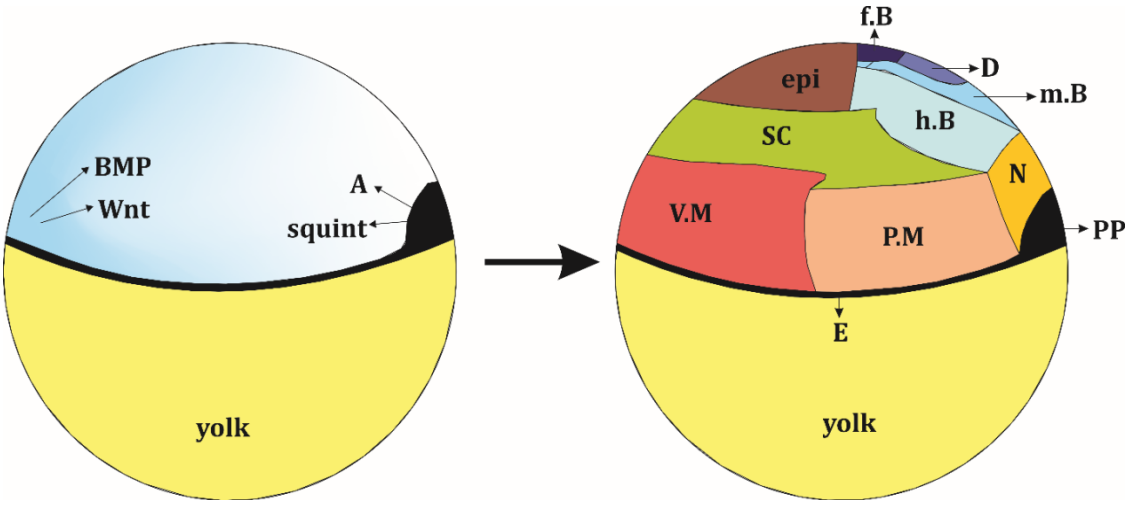

Figure 3: Morphogen gradients along the D/V axis pattern the germ layers (A: Antagonists of ventralizing factors; f.B: fore Brain; D: Diencephalon; m.B: mid Brain; h.B: hind Brain; N: Notochord; epi: epidermis; SC; Spinal Cord; V.M: Ventral Mesoderm; P.M: Paraxial Mesoderm: PP: Pre-chordal Plate; E: Endoderm).

In this regard, the dorsal-most cells transform into the anterior-most structures, such as the pharynx, whereas the lateral and ventral-most ones form the esophagus-stomach and intestinal cells, respectively (De Robertis, 2009; Thisse *et al*., 2000a, b; Munoz-Sanjuan and H-Brivanlou, 2001).

The A/V axis patterning leads to germ layer formation

The Functional equivalent of Xenopus laevis Nieuwkoop center in zebrafish

According to the cellular and molecular evidence, the functional equivalent of *X. laevis* Nieuwkoop center in zebrafish may be distributed between the YSL (namely the continuous organizer) and the dorsal marginal blastomers (namely the dorsal-blastula Spemann organizer) (Feldman *et al*., 1998; Schier, 2001). The β-cat, Nanog, maternal transcripts of the Squint itself, z-DVR1, and Goosecoid trigger the zygotic Squint expression at the dorsal organizer and YSL. However, its expression at the ventral region of the YSL chiefly depends on the Nanog. The zygotic expression of both Squint and Cyclops in the dorsal side of the embryo firstly depends on the maternal B-cat and secondly maternal/zygotic Nanog, which promotes B-cat effects (Schier, 2001; Bennett *et al*., 2007; Xu *et al*., 2012). In this regard, Nanog and β-cat induce the *mxtx2* encoding, which in turn transcribes the *cyclops*. While in the ventrolateral region, primarily Nanog and probably some unidentified maternal transcripts, assembled in the YSL, provoke both the *squint* and *cyclops* transcription, thereby inducing the ventrolateral mesendoderm formation in the overlying cells at the embryonic equator. In contrast, the dorsal mesendoderm genes are still expressed in the absence of YSL activity, because the dorsal blastula organizer is the major executive manager at the dorsal region (Fig. 3) (Liang and Rubinstein, 2003; Xu *et al*., 2012).

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Concentration gradients of Squint/Cyclops and their functions

Squint and Cyclops emanating from the dorsal organizer and YSL, redundantly create two concentration gradients, the first gradient from the equator (YSL region) to the animal pole and the second one from the dorsal organizer to the ventral region. The primary gradient participates in the A/P axis patterning of the organizer and germ layers formation. The second one involves the dorsal specification and A/P axis patterning of the organizer in coordination with the first gradient (Pogoda *et al*., 2000; Liang and Rubinstein, 2003; Schier and Talbot, 2003; Ulrich and Heisenberg, 2004; Xu *et al*., 2012).

In fact, due to the dynamic expression patterns of the Activin-like factors during embryonal development, and their nature as morphogens, these ligands may have such different roles (Chen and Schier, 2001; Thisse *et al*., 2000a; Hasanpour and Eagderi, 2020; Hasanpour *et al*., 2020, 2021a, b). The decreasing gradient of Squint toward the Animal pole determines the endoderm, mesoderm, and ectoderm, respectively. According to this hypothesis, higher and lower levels of Squint signaling lead to the formation of endoderm and mesoderm, respectively, and the principal absence of Squint signaling allows for ectoderm specification. In support of this hypothesis, various genetic and biochemical manipulations that lead to the progressive lowering of the Squint signaling preferentially affect the endoderm formation but retain most of the mesoderm tissues (Feldman *et al*., 1998; Dougan *et al*., 2003; Garnett *et al*., 2009; Tuazon and Mullins, 2015).

Principally, the basal level of the Activin-like factors activates the Erk (the Fgf-specific transducer) to sustain the stemness. Following their prompt enhancement, Erk directly and indirectly promotes the mesoderm formation in the dorsal region and antagonizes the endoderm formation locally. While high dose of the Activin-like factors leads to high levels of the Mixer/Mezzo and progressively Sox32, which in turn provoke the endoderm-specific markers expression (Schier, 2001; Ober *et al*., 2003; Tam *et al*., 2003; Poulain *et al*., 2006; Garnett *et al*., 2009; Tuazon and Mullins, 2015).

Mesoderm induction in the dorsal region

As discussed above, the prompt enhancement of the Erk directly and indirectly promotes the mesoderm formation in the dorsal region and antagonizes the endoderm formation locally by: (1) Fgf/Erk cascade disrupts the Sox32 (casanova) and Smad2/3 by phosphorylating, in turn, the Sox17 expression, a specific endoderm marker decreases. Additionally, Fgf/Erk decreases the Sox32 expression as well. (2) Fgf signaling alleviates the activity of Activin-like factor receptors (Taram/Alk4/ActRI), to simulate their attenuation. In other words, since lower amount of the Squint is sufficient to induce the mesoderm, its attenuation by Fgf evokes the positional information of the mesoderm. (3) Fgf signaling directly elicits the mesodermal markers expression, including the T-box

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transcription factors (Notail and Tbx-16) (Kodjabachian *et al*., 1999; Poulain *et al*., 2006; Mizoguchi *et al*., 2006; Garnett *et al*., 2009). On the other hand, Fgf signaling directly and indirectly (through Chordin) suppresses the Bmps expression and progressively the mesoderm formation. Therefore, Fgf adjusts the mesoderm formation through the Bmps reduction in the dorsal region (Kodjabachian *et al*., 1999; Mizoguchi *et al*., 2006; Poulain *et al*., 2006) (Fig. 2).

Mesoderm induction in the ventral region

High level of the Bmps promotes the mesoderm formation in the ventral region by the foxi1 expression, which is the suppressor of the Sox17. Taken together, the Bmp and Fgf/Erk pathway cooperate to restrict the number of endodermal progenitors induced in response to the Squint signaling (Kodjabachian *et al*., 1999; Poulain *et al*., 2006; Mizoguchi *et al*., 2006; Garnett *et al*., 2009).

Endoderm formation

High levels of the Activin-like factors are required for the endoderm specification. Principally, Squint activates expression of the sur (Fast/ FoxH1), which has a pivotal role in the Squint auto-regulatory feedback loop and control of the duration and intensity of its signaling. In addition, Smad2/3, by interacting with Fast/FoxH1, promotes the expression of genes that interfere with mesendoderm induction. Nevertheless, Fast/FoxH1 is not necessary to induce the mesendodermal genes, because the Maternal-Zygotic (MZ) *sur* mutants, in contrast to the Squint mutants, possess both the endoderm and mesoderm (Pogoda *et al*., 2000; Liang and Rubinstein, 2003; Grapin-Botton, 2009).

Principally, Smad2/3 singly or in combination with the Fast/FoxH1, regulates the transcription of *bonni & clyde* (Mixer and Mezzo) and *faust* (Gata5), which in turn provoke the *casanova* (Sox32) expression. Only the endodermal progenitors express *casanova*, because its ectopic expression at the presumptive mesendodermal cells transfate them into the endoderm (Pogoda *et al*., 2000; Liang and Rubinstein, 2003; Tam *et al*., 2003; Dougan *et al*., 2003; Poulain *et al*., 2006). The *bon* and *faust* mutants display 90% and 40% endoderm truncation, respectively. The *bon* and *faust* double mutation exacerbate the defects than either mutant alone. In addition, the *bon* is expressed normally in the *faust* mutant and inverse, confirming that the bon and *faust* act in parallel to endoderm formation. On the other hand, overexpression of the *bon* & *faust* in the *casanova* mutant embryos fails to repair the endoderm formation (Ober *et al*., 2003; Tam *et al*., 2003; Gong and Korzh, 2004; Zhiyuan and Vladimir, 2004; Poulain *et al*., 2006). Therefore, the *bon* (Mixer and Mezzo) and *faust* (Gata5) in parallel with each other act in the upstream of the *casanova*. Furthermore, *casanova* is necessary for the endoderm development, because *casanova* mutants lack all the endoderm progenitors.

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Additionally, its overexpression induces the Sox17 transcription, indicating that *casanova* autonomously acts as an upstream regulator of the Sox17 (and FoxA2) and induces its expression in a Squint-independent manner. Sox17, a transcription factor with a high mobility group (HMG) domain, is an intrinsic regulator of the endoderm formation and therefore it is considered a definitive endoderm marker (Tam *et al*., 2003; Gong and Korzh, 2004;Poulain *et al*., 2006; Grapin-Botton, 2009).

Ectoderm formation

Ectoderm, as the last germ layer, is formed in response to the lowest amount of all signaling factors, including the Wnts, Fgfs, Bmps, and Squint (Ulrich and Heisenberg, 2004; Tuazon and Mullins, 2015).

A/P axis patterning

Squint, Bmps, Wnts, Fgfs, and Retinoic acid signals are involved in the A/P patterning of the nervous system, wherein the forebrain develops in the absence of them; this model is termed the default model for neural induction. These ligands caudalize the neural tissues. For instance, the mutations affecting each of them cause an expansion of the anterior neural fates and a reduction of the tail and trunk (Thisse *et al*., 2000b, Schier, 2001; Tam *et al*., 2003; Tuazon and Mullins, 2015). In order to form the A/P gradient of the mentioned above ligands, the first cells migrating away from the margin constitute the prechordal plate, which will give rise to the anterior mesendodermal tissues such as the hatch gland, pharyngeal endoderm, and head muscles. The prechordal plate as a coherent group of cells beneath the ectoderm crawls toward the animal pole (anterior progenitor) (Kodjabachian *et al*., 1999, Schier, 2001, Thisse *et al*., 2000b). This structure and its movement evoke the AVE (Anterior Visceral Endoderm) and its anteroposterior migration in mice, as both possess the same markers, such as Cerberus, HesX1 (the anterior neuroectoderm marker), Lim1 (lhx1), Otx2, and Goosecoid. Bozozok and Squint cooperatively activate expression of the anteriorizing factors (i.e., Bmp, Wnt, Squint, and Fgf antagonists) in the prechordal plate (Liang and Rubinstein, 2003; De Robertis, 2009; Tuazon and Mullins, 2015). These anteriorizing factors are as follows: Spry2/4, Dkk1, Chordin, Noggin, Cerberus (Squint and also Bmp and Wnt antagonist), Follistatin1/3/5, and s-FRPs (Dougan *et al*., 2003; Bartscherer and Boutros, 2008). Following the secretion of anteriorizing factors, the A/P gradient of the Fgf, Wnt and Bmps will be formed (Feldman *et al*., 1998, Thisse *et al*., 2000b).

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