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Review

Symmetry Breaking and Establishment of Dorsal/Ventral Polarity in the Early Sea Urchin Embryo

Vincenzo Cavalieri [1,2,*] and Giovanni Spinelli [1,*]

[1] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze Edificio 16, Palermo 90128, Italy

[2] Mediterranean Center for Human Health Advanced Biotechnologies (CHAB), University of Palermo, Viale delle Scienze Edificio 16, Palermo 90128, Italy

* Authors to whom correspondence should be addressed; E-Mails: vincenzo.cavalieri@unipa.it (V.C.); giovanni.spinelli@unipa.it (G.S.); Tel.: +39-091-238-97408 (V.C.); +39-091-238-97400 (G.S.).

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Abstract: The mechanisms imposing the Dorsal/Ventral (DV) polarity of the early sea urchin embryo consist of a combination of inherited maternal information and inductive interactions among blastomeres. Old and recent studies suggest that a key molecular landmark of DV polarization is the expression of *nodal* on the future ventral side, in apparent contrast with other metazoan embryos, where *nodal* is expressed dorsally. A subtle maternally-inherited redox anisotropy, plus some maternal factors such as SoxB1, Univin, and p38-MAPK have been identified as inputs driving the spatially asymmetric transcription of *nodal*. However, all the mentioned factors are broadly distributed in the embryo as early as *nodal* transcription occurs, suggesting that repression of the gene in non-ventral territories depends upon negative regulators. Among these, the Hbox12 homeodomain-containing repressor is expressed by prospective dorsal cells, where it acts as a dorsal-specific negative modulator of the p38-MAPK activity. This review provides an overview of the molecular mechanisms governing the establishment of DV polarity in sea urchins, focusing on events taking place in the early embryo. Altogether, these findings provide a framework for future studies aimed to unravel the inceptive mechanisms involved in the DV symmetry breaking.

Keywords: dorsal/ventral axis; redox gradient; hypoxia; symmetry breaking; organizing centre; Nodal; Hbox12 transcription repressor; p38 MAPK; Wnt; sea urchin embryo

1. Introduction

During development of bilaterian embryos symmetry breaking is imposed through establishing of distinct polarities, which are precursors of the larval axes. Identifying the molecular mechanisms underlying the initial symmetry breaking is still now an overriding goal in understanding how nature is able to produce organisms with consistent and distinct anatomies.

In indirectly developing sea urchins, embryonic patterning along the dorsal/ventral (DV) axis, also known as oral/aboral axis, has been extensively studied in various species. In contrast to vertebrates, there is no developmental relationship between the embryonic DV axis and the structures of the body plan formed along the adult DV axis, which arises within the imaginal rudiment during the post-embryonic larval metamorphosis [1–3].

Irreversible establishment of the embryonic DV axis does certainly not occur prior to fertilization, nor does it display any consistent relation to the point of sperm entry [4–6]. Classical experimental embryology investigations surveying several sea urchin species indicated that cleavage *per se* does not play a causal role in the establishment of the DV axis. In fact, although there is a predominant association between the plane of the first cleavage division and the future DV axis in *Strongylocentrotus purpuratus* [7], the orientation of the first cleavage furrow and DV axis differs widely between species, and even amongst the embryos of a single individual [8–12].

More than one century ago, the seminal bisection experiments of Driesch testifying the totipotency of two- and four-cell stage sea urchin blastomeres [13], clearly indicated that the DV axis is not firmly established in the unfertilized egg. Rather, it is progressively specified during early cleavage through a conditional process that relies on a combination of inherited maternal information and inductive interactions among blastomeres [14–16]. Nodal signaling provides a fundamental driver input for specifying DV asymmetries [17,18], although, differently with respect to other metazoan embryos, in the sea urchin Nodal operates on the ventral rather than on the dorsal side.

DV polarity becomes morphologically recognizable from the onset of gastrulation, when the embryo begins to flatten on the ventral side, and two bilaterally symmetric thickenings of the ectoderm form on ventrolateral regions (Figure 1). In the intervening time, subpopulations of primary mesenchyme cells migrate within the blastocoel cavity guided by signals emitted by these regions, settle into two ventrolateral aggregates adjacent to the ectoderm thickenings, and eventually mineralize two skeletal primordia [19–21]. At the end of gastrulation, the opening of the larval mouth occurs by bending of the archenteron towards the ventral ectoderm and subsequent fusion of the two epithelia. At the pluteus stage (Figure 1), the ectoderm is noticeably partitioned into four main domains along the DV axial coordinates: (1) the ventral/oral ectoderm, a thickened epithelium surrounding the mouth; (2) the dorsal/aboral ectoderm, a squamous epithelium that covers most of the rest of the larval body; (3) the ciliary band, a belt of ciliated cells positioned at the border between ventral and dorsal ectoderm; and (4) the apical neurogenic domain. Furthermore, the dark-red pigment cells, derived from secondary mesenchyme precursors, can be easily recognized by their dispersed disposition embedded within the dorsal, but not the ventral, ectoderm layer (Figure 1).

With careful examination, one can detect many earlier signs of DV asymmetries and there are now a number of early molecular indicators of these properties.

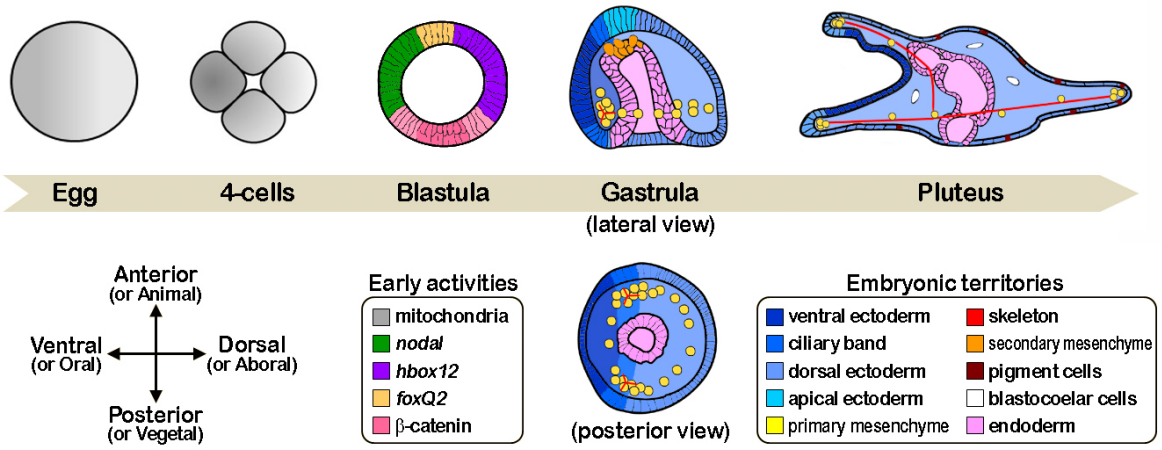

Figure 1. Simplified scheme depicting key developmental stages and early molecular activities regulating morphogenesis along the DV axis of the sea urchin embryo. See text for details.

2. Symmetry Breaking: Subtle Redox Anisotropies Prefiguring the DV Axis

The first molecular manifestation that foreshadows DV polarity occurs during the initial cleavage stages, consisting in a redox gradient sustained by mitochondrial cytochrome oxidase activity [22,23]. More precisely, high redox potential, and the resulting oxidizing environment, significantly correlates with ventral fate, although this juxtaposing association is not statistically observed in 100% of cases [24–26].

Pioneering evidence highlighted that DV polarity can be biased by exposing embryos to hypoxic condition or respiratory inhibitors [22,27]. More recent studies in *S. purpuratus* also indicated that DV polarity is similarly assigned if embryos are cultured in immobilized tight clusters, wherein a redox gradient forms across the inside-outside axis of the cluster [24]. Additional pieces of information highlighted that such a redox gradient relies upon the maternally-derived uneven distribution of mitochondria (Figure 1), and that disruption of such an anisotropy, by either centrifugation of eggs (which displaces mitochondria toward the centrifugal pole) or injection of purified mitochondria, can entrain DV polarization [25]. In support of these findings, it has been shown that treatment with compounds, such as Cobalt (Co(II)), that in other systems stimulate the generation of uniform high levels of reactive oxygen species (ROS) perturbs DV patterning of the resulting embryos, most likely altering a necessary redox asymmetry [28–30].

Indeed, the asymmetrical mitochondrial distribution in the *S. purpuratus* embryo correlates with both differential intracellular ROS levels, mainly in the form of H_2O_2, and *nodal* expression [26]. In fact, quenching mitochondrial H_2O_2 emissions by clonal overexpression of mitochondrially-targeted catalase entrains DV polarity by under-expression of *nodal* [26]. Similarly, embryos cultured under hypoxic conditions through early cleavage exhibit significantly decreased levels of H_2O_2 and develop as radialized larvae lacking DV polarity [25,31]. Worth mentioning, *nodal* expression is not abolished in these larvae, but rather its spatial localization to one side of the embryo is prevented by hypoxia, revealing that redox anisotropies are not required to activate *nodal* transcription by itself, but rather to provide an initial spatial bias in the rate of *nodal* transcription [32].

A possible link between redox gradient and *nodal* expression has been postulated by the known function of the p38 MAP kinase signaling pathway in the transcriptional responses downstream of oxidative stress in several metazoans [33–36]. Formerly identified in *Lytechinus variegatus* as a kinase whose activity is required for *nodal* expression [37], p38 has been subsequently demonstrated to be responsive to ROS in *S. purpuratus* embryos [26]. Further downstream, p38 is thought to activate the maternal redox-sensitive bZIP and Oct1/2 factors only on the ventral side of the early embryo, reflecting the initial respiratory anisotropy into a polarized transcriptional regulatory state [37–40]. Importantly, binding sites for the mentioned factors have been identified in the *cis*-regulatory module controlling the initiation of *nodal* gene transcription [38,39], strongly suggesting that redox signaling influence DV patterning through *nodal*.

3. DV Polarity Establishment: Early Positive and Negative Activities Shaping the *Nodal*-Expressing Organizing Centre

The genetic landmark of polarization along the secondary axis is the zygotic expression of Nodal, a member of the TGF-β superfamily. Previous studies in *Paracentrotus lividus* and *S. purpuratus* species shown that *nodal* is first transcribed broadly, at an extremely low level, all around the prospective ectoderm of the 32/60-cell stage embryo [17,18,40]. Afterwards, *nodal* expression is rapidly downregulated in the presumptive dorsal blastomeres, exclusively marking the prospective ventral ectoderm of the early blastula [17,18,40]. Not only *nodal* expression is required for specification of the latter territory, but the small group of cells that specifically express *nodal* at the early blastula stage, thereupon behaves as an organizing centre imposing DV polarity in all three germ layers of the embryo [17,18,41–43].

As mentioned in the previous section, the p38 signaling pathway could bridge the redox gradient to *nodal* expression. Although being globally active during embryogenesis of the sea urchin, p38 becomes transiently inactivated in dorsal blastomeres, *viz* at the low end of the respiratory gradient, just before the onset of *nodal* expression [37]. Moreover, inhibition of the p38 function abrogates *nodal* transcription and, in turn, disrupts DV polarization [37]. According to the mentioned model, at the early blastula stage the maternally related anisotropy in redox gradient would transiently inactivate the p38 kinase in the future dorsal ectoderm [26,37], somehow leading to the activation of predicted redox-sensitive transcription factors on the ventral side [38–40]. The *cis*-regulatory apparatus of *nodal* responds to these factors, as well as to the maternal positive inputs of SoxB1 and Univin (another TGF-β family member), directing the expression of the gene within a discrete sector of the ectoderm that exactly corresponds to the presumptive ventral ectoderm [17,18,38,39,44].

DV axis formation and *nodal* expression are also dependent on functional Wnt signaling emanating from the posterior, or vegetal, pole of the cleaving embryo [17]. Nuclearization of β-catenin in posterior blastomeres allows the interaction with TCF/Lef transcription factor to regulate downstream gene expression and, in turn, expands the β-catenin signal to adjacent cell layers in a posterior to anterior direction [45–48].

Dissection of the *cis*-regulatory apparatus of the *nodal* gene identified a TCF/Lef binding site that is required for proper expression of a promoter-reporter transgene [39]. However, direct association of TCF/β-catenin heterodimer to this site has not been mapped so far, probably because the nuclearization

of β-catenin is not detected in cells of the anterior, or animal, hemisphere [46,49]. Most probably, β-catenin regulates *nodal* transcription not directly but, rather, through an unidentified signal originating from posterior cells. In addition, although blocking the nuclearization of β-catenin represses *nodal* expression at the blastula stage [17], the initial transcription of *nodal* is not affected [50], indicating that such a vegetal signal acts on the maintenance of *nodal* expression rather than the initial activation.

Although all of the known positive inputs activating *nodal* transcription are present maternally and very broadly distributed in the early embryo, ectopic expression of *nodal* beyond the ventral ectoderm founder cells is specifically hampered by tissue-specific negative regulators. In this regard, we have shown in *P. lividus* that the Hbox12 homeodomain repressor is expressed by prospective dorsal ectoderm cells, spatially facing and preceding the onset of *nodal* transcription, where it acts preventing the ectopic activation of *nodal* expression [51–54]. Functional analysis revealed that expression of *hbox12* and *nodal* genes are mutually exclusive and both required for DV polarization. In fact, overexpression of Hbox12 specifically attenuates *nodal* transcription, while loss of Hbox12 function allows broad ectopic expression of *nodal*, and in both the experimental assays the resulting embryos do not acquire DV polarity [53,54]. Intriguingly, Hbox12 is functionally upstream of p38, being specifically involved in the transient inactivation of the kinase in dorsal blastomeres [53]. Therefore, not only *hbox12* is the earliest known zygotic gene differentially expressed along DV axis, but it also represents the foremost negative regulator allowing competence for spatial positioning of the DV organizer.

FoxQ2, another transcription repressor, also contributes to prevent the spatial spreading of *nodal* expression in the animal hemisphere. Expression of *foxQ2* begins slightly earlier than that of *nodal*, initially in all animal blastomeres, driven by the Six3 transcription activator [50,55,56]. Soon thereafter, posterior Wnt1 and Wnt8 signals act in parallel to progressively restrict *foxQ2* transcription in the apical neurogenic ectoderm [50,57,58]. This temporally coordinating mechanism prevents *nodal* expression from prematurely reaching high levels. However, although overexpression of *foxQ2* efficiently abrogates *nodal* transcription, the opposite effect on *nodal* in the animal plate is obtained only when the functions of FoxQ2 and Lefty (see below) are doubly knocked down, suggesting a functional cooperation between the two negative regulators of Nodal signaling [50].

A further attractive regulator potentially involved in the spatial control of *nodal* expression is the Myb transcription factor. Although the spatial distribution of Myb in the early embryo has not yet been determined, Myb is a redox-sensitive factor [59,60] known to act as a repressor in ventral territories of the sea urchin embryo [61]. This evidence has been provided by the *cis*-regulatory analysis of the *CyIIIa* and *hbox12* genes, both beginning to be specifically transcribed in the presumptive dorsal ectoderm during early embryogenesis [52,61]. Surprisingly, the *cis*-regulatory apparatus of the *nodal* gene also contains a Myb-like consensus binding sequence that, when mutated in gene transfer assays, leads to increased reporter expression [39]. This opens the possibility that the Myb repressor might work to fine-tune the transcriptional output of the *nodal* gene in ventral cells.

4. DV Polarity Maintenance: How the Organizing Centre Works

Once the expression of *nodal* is initiated by early inputs, the peculiar ventral localization of the *nodal*-expressing domain is probably consolidated by a reaction-diffusion system, although direct evidence have to be provided in the sea urchin. In fact, the best direct evidence for it comes from a recent

study in zebrafish, where the effective diffusion coefficients of GFP-tagged TGF-β proteins were measured during embryogenesis [62]. The reaction-diffusion system would involve a positive feedback loop related to the short-range Nodal signal transduction system, and a concurrent Nodal-dependent mechanism given by the production of the Nodal antagonist Lefty [17,38,63,64]. With respect to the former facet, experimental confirmation comes from *cis*-regulatory studies, since mutation, in the promoter of *nodal*, of sequences postulated to be targeted by Smad2/3, a transducer of Nodal signaling [26,65], reduces the reporter expression in transgenic embryos [38,39].

Lefty and Nodal are produced by the same cells, albeit the former is thought to diffuse more rapidly, thus acting as a long-range Nodal inhibitor [17,63,64]. In accordance with this hypothesis, DV axis and *nodal* expression are both lost in embryos in which Lefty is overexpressed. By contrast, impairing Lefty function converts most of the embryonic territories toward a ventral fate through ectopic expression of *nodal* [41,63].

Direct targets of Nodal signaling within the ventral ectoderm also include genes encoding the TGF-β pathway extracellular components BMP2/4 and Chordin [17,66–70]. The BMP2/4 ligand acts as a diffusible relay molecule to specify the dorsal ectoderm, to which its signaling activity is confined, due to inhibition of BMP2/4 reception by Chordin within the ventral ectoderm [17,67,69,71]. This model, which is also supported by computational simulation [72], explains why in the absence of *nodal* expression, not only the specification of ventral structures is abolished, but the differentiation of dorsal territories is suppressed as well [17].

It must to be also taken into account that signaling events require differential migration of all the mentioned cytokines across the fibrous mesh of the extracellular matrix that surrounds and support cells. In such a milieu, proteoglycans has been suggested to play fundamental roles in the differential localization and stability of TGF-β ligands during axial patterning of various metazoans, including sea urchins [69,73–76]. Likewise, a recent study indicates that dynamin-mediated endocytosis limits the range of Nodal diffusion in *S. purpuratus* embryos [77].

The amount of details available on molecular circuits that govern DV patterning of the embryonic territories downstream of *nodal* expression is growing rapidly and, so far, more than fifty genes are known to be regulated, either directly or indirectly, by Nodal signaling [44,70,71,78–80]. Large scale studies began to describe the epistatic relationships among these genes [44,81], highlighting that sequential interplay between inductive and suppressive events controlling Nodal signaling is critical for DV patterning. In this regard, *wnt1* signaling from the posterior pole suppresses *nodal* transcription in posterior ventral cells during gastrulation, contributing to sculpt the spatial expression pattern of *nodal* even at later stages [82]. In *wnt1* morphants the *nodal* expressing territory is expanded posteriorly in the ventral side, provoking DV patterning defects that include a ventral to dorsal shift in the position of the posterior ciliary band [82]. This finding reveals that there is a continuing requirement to restrict Nodal signaling throughout development.

5. Conclusions and Future Perspectives

While DV polarization is morphologically not apparent in the sea urchin zygote, axial specification is accomplished at the molecular level in the early embryo. Deciphering the molecular hierarchies that govern the establishment of DV axis provides explanation of how the spatially asymmetric expression

of regulatory genes is gradually translated into the anatomically distinct structures of the embryo. The critical molecular events discussed in the previous sections are diagrammatically summarized in Figure 2. Although the substantial progress earned in the last decade of experiments, since the role of *nodal* began to be characterized in sea urchins, a number of fuzzy points still need to be clarified.

How the labile redox anisotropies in the egg and early embryo are reflected into polarized gene expression patterns remains a central but largely enigmatic issue. Functional analysis revealed that Hbox12 is required for the transient inactivation of the p38 kinase in dorsal blastomeres, raising the question of whether *hbox12* gene expression and/or function are directly regulated by the redox state. Being *hbox12* expression achieved in the reducing environment specifically associated to the dorsal side of the early embryo, a likely candidate involved in the activation of *hbox12* transcription could be HIF1α. This hypothesis deserves further investigation, being supported by recent studies showing that the HIF1α transcription activator is stabilized in a reducing environment [83,84], and by the fact that knock-down of HIF1α function strongly reduces the expression of dorsal-specific markers in the very early sea urchin embryo [85].

In an alternative scenario, inter-specific differences could exist in the initial mechanisms used to specify DV polarity. In fact, while in recent years the role of the mitochondrial gradient and redox signaling have been firmly established in *S. purpuratus*, they have not been rigorously investigated in any other species. However, threaded between the lines of a recent paper, it is mentioned that experimental manipulations that perturb the redox gradient have very modest effects on the spatial expression of *nodal* in *P. lividus* [44]. In light of this, it remains possible that redox polarization represents a species-specific mechanism.

Actually, given the robustness of the reaction-diffusion device, all that is needed for the Nodal-Lefty genetic circuit to break DV symmetry is any of a number of alternative stochastic means that generate an initial spatial bias in *nodal* transcription along the secondary axis. Thus, *S. purpuratus* may use mitochondrial signaling, while other species may utilize, for instance, localization of a transcription factor.

In this regard, the requirement for Hbox12 in DV axis specification has thus far only been established in the *P. lividus* species [53,54]. Available evidence indeed indicates that there is no *hbox12* orthologue in *S. purpuratus*, and that the closest homolog of *hbox12* in the latter species is *pmar1* [51,53], which is instead involved in micromere lineage specification [86]. This strongly suggests that DV axis specification in *S. purpuratus* cannot depend on *hbox12*, since the gene probably does not exist in that species. Hence, there is the very captivating possibility that the mechanism of initial axis specification is completely different between *S. purpuratus* and *P. lividus* species. In other words, the former utilizes redox polarization to achieve the initial localization of *nodal* activity, while the latter requires localized transcriptional activation of Hbox12, independently of redox signaling.

This hypothesis is advocated by the well-fitting clue that the two sea urchin species actually differ in some aspects of DV axis determination. In particular, in *S. purpuratus* the DV axis passes through a plane about 45° clockwise from the first cleavage furrow [7], indicating that secondary axis specification is initiated between fertilization and first cleavage, which is consistent with the asymmetric distribution of mitochondria within eggs and early embryos of this species [25]. Classical studies in *P. lividus* embryos instead demonstrate that DV axis is randomly oriented with respect to the first cleavage plane, and that it is established between the fifth and eighth cleavage [8], which broadly corresponds to the peak of *hbox12* transcription [51,53].

Whatever is the mechanism providing the initial bias of *nodal* activity in the early embryo, it most likely reverberates on the differential modulation of p38 activity. Therefore, it will be important to determine not only whether p38 directly phosphorylates the predicted bZIP and Oct1/2 downstream targets, but also whether or not the phosphorylation status influences the activity of the mentioned transcription factors in the sea urchin embryo. Albeit investigation on Oct1/2 recently began [40], the exact identity of the bZIP transcription factor, among those conserved in the echinoderm genomes, supposed to be involved in the redox transduction pathway is currently not known. No less important will be the mapping of the physical binding of the redox-sensitive transcription factors, including Myb, to the cognate *cis*-regulatory sequences predicted in the *nodal* promoter.

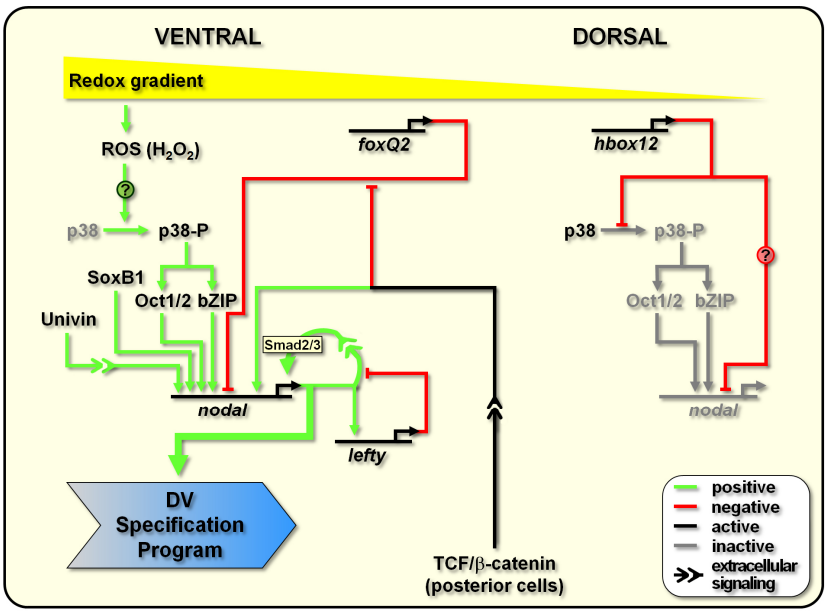

Figure 2. Diagram illustrating the early molecular events regulating the establishment of the DV organizing centre in the sea urchin embryo. See text for details.

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Conflicts of Interest

The authors declare no conflict of interest.

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