



# DISSERTATION | DOCTORAL THESIS

Titel | Title

An alternative mechanism to form an organizing center in  
mouse neural tube organoids

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of  
Doctor of Philosophy (PhD)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree  
programme code as it appears on the  
student record sheet:

UA 794 620 490

Dissertationsgebiet lt. Studienblatt | Field of  
study as it appears on the student record  
sheet:

Molekulare Biologie

Betreut von | Supervisor:

Elly Tanaka PhD



Für  
Antonia,  
Hilda und Franz.



## Acknowledgements

I would like to thank everyone who supported me throughout my PhD.

First, I would like to express my dearest acknowledgment to Prof. Dr. Elly Tanaka for her exceptional supervision, mentoring, and endless support. Her constant encouragement in all aspects of life, both private and work-related, her always open ear, her smart ideas, and her belief in me helped me identify my strengths and made me grow immensely as a scientist through our numerous engaging discussions.

I am also extremely grateful for my dear colleagues in the Tanaka lab, both past and present, who are an incredibly kind, open, and intelligent group of scientists. They were always there to give advice and provide scientific input, and they accompanied me in various experiences and events, becoming dear friends along this journey. Especially, I want to thank Elena C., Laura, Hannah, Katharina, Anja, Diego, Elena G., Sonja, Anastasia, Simone, Basti and Lucrezia. My thanks also go to Leo, Wouter, Pietro, Elad and Stephan for their scientific feedback.

I would also like to thank Prof. Dr. Christa Bucker and Prof. Dr. Dave Keays, my thesis advisors, for their scientific input and guidance throughout the years. Additionally, I am thankful to Dr. Eric Siggia for the engaging discussions and his trust in me. I further extend my deepest gratitude to all my appreciated collaborators and contributors from the Tanaka lab and beyond, who provided valuable assistance at various stages of my PhD, making the primary publication of my PhD a smooth and rewarding experience. I especially want to thank Dr. Ani Kicheva and her lab, as well as Dr. James Briscoe and his lab, for their valuable contributions and for the fruitful and interesting discussions.

Special thanks also go to Amina Zankel and Sylvia Vesely, who quickly helped me find my way as an at that time pregnant scientist. They made my six months working in the pregnancy lab a fun and easy journey, and I am immensely grateful for the opportunity and their help. I am further thankful to Pawel and Alberto from the BioOptics facility, who were always eager to assist in any situation, whether it was dealing with my numerous samples or generously re-locating the Viventis lightsheet microscope to the pregnancy lab to ensure a safe imaging environment. Gerald's help in sorting cells, along with our funny conversations about life, music, or our special names collection, were valuable for me. Additionally, I would like to acknowledge the entire MBS facility, especially Martina, Sabina, Markus, Robert and Zuzana, as well as the IT and NGS departments for their support.

However, not only were the people I met during my PhD incredibly important for this period, but also those from various other parts of my life. Especially, I owe my deepest gratitude to my family, Nicolas and Antonia, for being the rock that gave me balance and grounded me during this time. Antonia, my daughter and greatest love, brightened every day with her sweet, empathetic, and cheerful mind. Nicolas, for his calm and down-to-earth approaches that almost always managed to relax me and help me see everything from a wider perspective. My whole-hearted thanks go to my parents Hildegard and Erich, and grandparents Hilda and Franz for their unconditional love and support, not only during my PhD but throughout my entire life. My brother Pascal deserves a special remark for ensuring we regularly went out for dinner, especially to all-you-can-eat restaurants.

I am also immensely grateful to my very dear friends Steffi H., Steffi W., Sophie, Kathi, Meggy, Kerstin, Romina and Kristina, as well as the Ebi core gang — Elisabeth, Philipp and Peter — who have played important and constant roles in my life for decades already. Klara and Catarina, whom I met during my Bachelor's and Doctoral studies, respectively, have become great friends beyond our scientific environment, sharing knowledge and providing support. Steffi L. and Tom shared the suffering and conference moments with me. Noelia, Steph, Fanny and Andi, thank you for sharing the parenthood journey and the fun moments along the way. I am also deeply grateful to Dr. Ralph Neumüller for always believing in me from the very beginning, for his mentorship during my Master's, and for his constant support until now. Additionally, I thank my friends from Boehringer Ingelheim — Alex, Babsi, Kathi and Carina — for their friendship.

Lastly, I would like to thank the VBC PhD program coordinators, Christopher Robinson, Chiara Ceriotti, Eva Schmid, and Yan Sun, for their help throughout my PhD. Their efforts providing assistance and in organizing valuable courses on campus have significantly contributed to my knowledge in various scientific aspects.

I feel extremely lucky to have the opportunity to thank so many wonderful people. In summary, I am more than happy to have experienced all the incredible things and met amazing people during my PhD journey that I would not want to miss.

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## Abstract

The development of the embryo is a complex process orchestrated by the integration of tightly regulated mechanical as well as signaling cues. The neural tube, which is the precursor to the central nervous system (CNS) in vertebrates, has been a hallmark example of embryonic induction and patterning. It requires the establishment of two opposing signaling centers that correctly pattern the progenitor cells in the neural tube, leading to the generation of diverse neuronal cell types. The ventral signaling center, the floorplate, typically forms through local signals received from the underlying notochord, a mesodermal rod-like structure. However, neural tube organoids (NTOs) formed *in vitro* from pluripotent stem cells, can develop a fully functional floorplate through self-organization, independent of external structures like the notochord that provide spatial information. A key challenge in biology is to understand how stem cells form such regulatory organizers. This dissertation focuses on using NTOs to investigate the mechanisms underlying this notochord-independent floorplate self-organization. The first part of this work focusses on in-depth characterization of NTO formation, which gives knowledge of epithelialization, neural differentiation and dorsoventral pattern induction. In the second part of this work, this knowledge is applied to study how self-organization of a functional floorplate after a single global pulse of the signaling molecule retinoic acid (RA) occurs in NTOs. Initially, Forkhead box protein A2 (FOXA2)-positive (FOXA2<sup>+</sup>) floorplate cells start to be expressed in a spatially scattered manner, which then condense into multiple clusters that undergo competition and physical sorting before resulting in a final floorplate. Using a combination of sequencing, drug screening and pathway localization, diffusible molecules of the Bone morphogenetic protein (BMP) pathway were identified to govern long-range cluster competition. Additionally, mutating the BMP inhibitor *Noggin* disrupted floorplate formation in both, NTOs and the mid-hindbrain region of mouse embryos, suggesting redundant mechanisms for robustness. This work shows the mechanism by which a global RA pulse results in a single floorplate per organoid through inter-cluster competition. Furthermore, it highlights that defining mechanisms directing organizer self-organization is critical in exploiting the developmental plasticity of stem cells for directed tissue engineering.



## Zusammenfassung

Die Embryonalentwicklung ist ein komplexer Prozess, der durch die Integration streng regulierter mechanischer und über Signalwege übertragender Faktoren gesteuert wird. Das Neuralrohr, welches der Vorläufer des zentralen Nervensystems in Wirbeltieren ist, ist ein herausragendes Beispiel für die embryonale Induzierung und Regionalisierung. Es erfordert die Etablierung von zwei gegenüberliegenden Signalzentren, welche die neuronalen Vorläuferzellen im Neuralrohr richtig regionalisieren, was zur Entstehung verschiedener neuronaler Zelltypen führt. Das ventrale Signalzentrum, die Bodenplatte, bildet sich typischerweise durch lokale Signale, die vom darunter liegenden Notochord, einer mesodermalen stäbchenartigen Struktur, empfangen werden. Neuralrohrorganoide, die *in vitro* aus pluripotenten Stammzellen gebildet werden, können jedoch durch Selbstorganisation eine voll funktionsfähige Bodenplatte entwickeln, unabhängig von externen Strukturen, wie dem Notochord. Eine zentrale Herausforderung in der Biologie besteht darin, zu verstehen, wie Stammzellen solche regulatorischen Organisatoren bilden. Diese Dissertation fokussiert sich auf die Verwendung von Neuralrohrorganoiden zur Untersuchung der Mechanismen, die dieser Notochord-unabhängigen Selbstorganisation der Bodenplatte zugrunde liegen. Im ersten Teil dieser Arbeit wird die umfangreiche Charakterisierung der Neuralrohrorganoid-Bildung, Erkenntnisse über deren Epithelialisierung, die neurale Differenzierung und die Induktion dorsoventraler Regionalisierung behandelt. Im zweiten Teil dieser Arbeit wird dieses Wissen genutzt, um zu untersuchen, wie die Selbstorganisation einer funktionellen Bodenplatte nach einem Retinolsäure-Puls in Neuralrohrorganoiden erfolgt. Zunächst werden FOXA2-positive Bodenplattenzellen zufällig im Neuralrohrorganoid verteilt exprimiert, anschließend konzentrieren sie sich zu mehreren Clustern, die miteinander kommunizieren, konkurrieren und sich aufgrund physischer Merkmale sortieren, bevor sie eine endgültige, vollfunktionale Bodenplatte bilden. Durch eine Kombination aus Sequenzierung, Wirkstoffscreening und Lokalisierung der Signalwege wurden diffundierende Moleküle des BMP-Signalwegs identifiziert, die diesen Wettbewerb zwischen den Clustern über weite Strecken steuern. Darüber hinaus wurde durch die Mutation des BMP-Inhibitors *Noggin* die Bildung der Bodenplatte sowohl in den Neuralrohrorganoiden als auch in der Mittel- und Hinterhirnregion von Mausembryonen gestört, was auf redundante Mechanismen für Robustheit hindeutet.

Diese Arbeit erklärt den Mechanismus, durch den ein globaler Retinolsäure-Puls zu einer einzigen Bodenplatte pro Organoid durch Wettbewerb innerhalb der Cluster führt. Darüber hinaus unterstreicht sie die Wichtigkeit Mechanismen zu definieren, welche die Selbstorganisation von Organisatoren steuern, um die Entwicklungsplastizität von Stammzellen für Tissue Engineering zu nutzen.

# I. Introduction

## I.1 Overview

Organization is a key element of development. Creating organization during embryonic development is defined by precise mechanisms that orchestrate complex processes of development, growth, and differentiation in living organisms, from the molecular level to the entire organism. So-called organizers secrete signals to provide positional information and regulate gene expression, guiding cells to differentiate into specific fates and organize them to form tissues and organs (Spemann & Mangold, 1924; R. Harland, 2000, p. 200). One example of organization is neural induction (Stern, 2001, 2005). Neural induction is the main mechanism that creates organization to form neural tissue, including the neural tube, which is essential for nervous system development in the vertebrate species. The function of organizers involved in ventral neural tube formation and patterning, the node, notochord, and floorplate, has been well-studied. However, how these organizers are formed in the first place has been a topic of ongoing debate. Notably, recent research has shown that organizers can form *in vitro* through the process of self-organization from local or distant cell interactions, even without their embryonic inducers (Sato et al., 2009; Eiraku et al., 2011; A. Meinhardt et al., 2014; Martyn et al., 2018). Neural tube organoids (NTOs) are three-dimensional (3D) stem cell models mimicking early neural tube development (A. Meinhardt et al., 2014), thereby representing valuable tools to study self-organization and organizer formation in a defined context. Notably, NTOs can form a localized, fully functional organizer, the floorplate, through self-organization. This thesis aims to explore the underlying reasons and mechanisms behind organizer self-organization in NTOs.

## I.2 Creating organization in biology – Embryonic induction

*“Embryonic induction is an interaction between one (inducing) tissue and another (responding) tissue, as a result of which the responding tissue undergoes a change in its direction of differentiation. This is probably the single most important mechanism in vertebrate development leading to differences between cells and to the organization of cells into tissues and organs.” John B. Gurdon (Gurdon, 1987)*

Life begins from a single cell, the fertilized egg or zygote, which goes on a remarkable journey of cell division, differentiation, and organization in order to build various cells and tissues to form a complex, multicellular organism (Hiiragi & Solter, 2004; Louvet-Vallée et al., 2005). It divides rapidly until it breaks symmetry to form the blastocyst (Motosugi et al., 2005). In mammals, the blastocyst continues to divide and implants into the uterine wall, initiating gastrulation. During gastrulation in amniotes, a classification that includes birds and mammals, the primitive streak forms on the back side of the embryo's surface because of Nodal signaling between the extraembryonic tissue and the epiblast (Abercrombie & Waddington, 1937; P. P. L. Tam & Behringer, 1997). The primitive streak extends along the midline, establishing the anterior-posterior body axis (Downs & Davies, 1993; Downs, 2009), which induces gastrulation (Chuai et al., 2006). Epiblast cells migrate toward the primitive streak and subsequently ingress through it, a process that involves cell migration and re-arrangement. This leads to the formation of the three primary germ layers: ectoderm (outer layer), mesoderm (middle layer), and endoderm (inner layer). The nervous system then develops from the ectoderm during a process called neural induction.

### I.3 Neural induction

Neural induction is the process during gastrulation where the ectoderm, the outer layer of the early embryo, is induced to become neural tissue (Chitnis & Kintner, 1995; Crunz, 1997; Stern, 2001, 2005). This tissue ultimately gives rise to the CNS, which consists of the brain and spinal cord, and defines the neural crest, which becomes the peripheral nervous system PNS (Kandel et al., 2000). This process is initiated by the formation of a specific region within the embryo, the organizer, which secretes signals that influence the fate of surrounding cells (Spemann & Mangold, 1924; R. Harland, 2000, p. 200) (**Figure 1**).

To direct neural activity, the organizer secretes signaling molecules such as Noggin (Lamb et al., 1993; W. C. Smith & Harland, 1992), Chordin (Piccolo et al., 1996; Sasai et al., 1995) and Follistatin (Hemmati-Brivanlou et al., 1994), thereby inhibiting BMPs which would otherwise promote the formation of epidermal instead of neural tissue from the ectoderm (R. M. Harland, 1994; Sasai & De Robertis, 1997). This process creates a region of ectoderm that will develop into neural tissue, the neural ectoderm (Sasai & De Robertis, 1997; Stern, 2001,

2005). The necessity of BMP inhibition in neural induction was shown by experiments performed in isolated explants from different positions of the animal cap of early frog embryos (Grunz & Tacke, 1989; Hemmati-Brivanlou & Melton, 1997; P. A. Wilson & Hemmati-Brivanlou, 1995). The explants differentiated into neural tissue when dissociated and cultured *in vitro*. Interestingly, when explants were isolated but not dissociated, this led to the formation of epidermis instead. However, in the presence of BMP inhibitors the explanted and dissociated tissue differentiated into neural tissue, while their exposure to BMP4 protein lead to differentiation into epidermal tissue (Hemmati-Brivanlou & Melton, 1997; P. A. Wilson & Hemmati-Brivanlou, 1995). Also the re-aggregation of dissociated explants lead to their differentiation into neural cells (Grunz & Tacke, 1989), suggesting that endogenous BMP signaling can induce epidermal fates in intact ectoderm (P. A. Wilson & Hemmati-Brivanlou, 1995).

The inhibition of BMP, respectively transforming growth factor- $\beta$  (TGF $\beta$ ), signaling in the ectoderm is seen as the default model for neural induction also in mammalian species (Ybot-Gonzalez et al., 2007), and further studies demonstrating similar mechanisms can promote neural development also in pluripotent mouse and human embryonic stem cells (ESCs) in culture, suggesting that this molecular mechanism is preserved across various species (Hamburger, 1988; Ozair et al., 2013).

Once formed, the neural ectoderm thickens to form the neural plate, a flat sheet of cells located on the dorsal side of the embryo (**Figure 2A**). Afterwards, the edges of the neural plate rise and fold towards each other, leading to the formation the neural groove. Eventually, the edges of the folding neural plate fuse to form the neural tube, which is the precursor of the CNS. This process is called neurulation and can vary spatiotemporally between species (Golden & Chernoff, 1993; Copp & Greene, 2010). During neurulation, the embryo's body axis extends caudally due to a bipotent cell population located in the primitive streak-associated epiblast, the neuromesodermal progenitors (Henrique et al., 2015; Gouti et al., 2015), which contribute to both neural tube elongation and the adjacent paraxial mesoderm as the body axis extends. These cells are balanced by Wingless/Integrated (WNT), fibroblast growth factor (FGF) and RA signaling, with WNT/FGF promoting a mesodermal fate, while RA induces neural differentiation (Martinez Arias & Steventon, 2018; Sambasivan & Steventon, 2021).

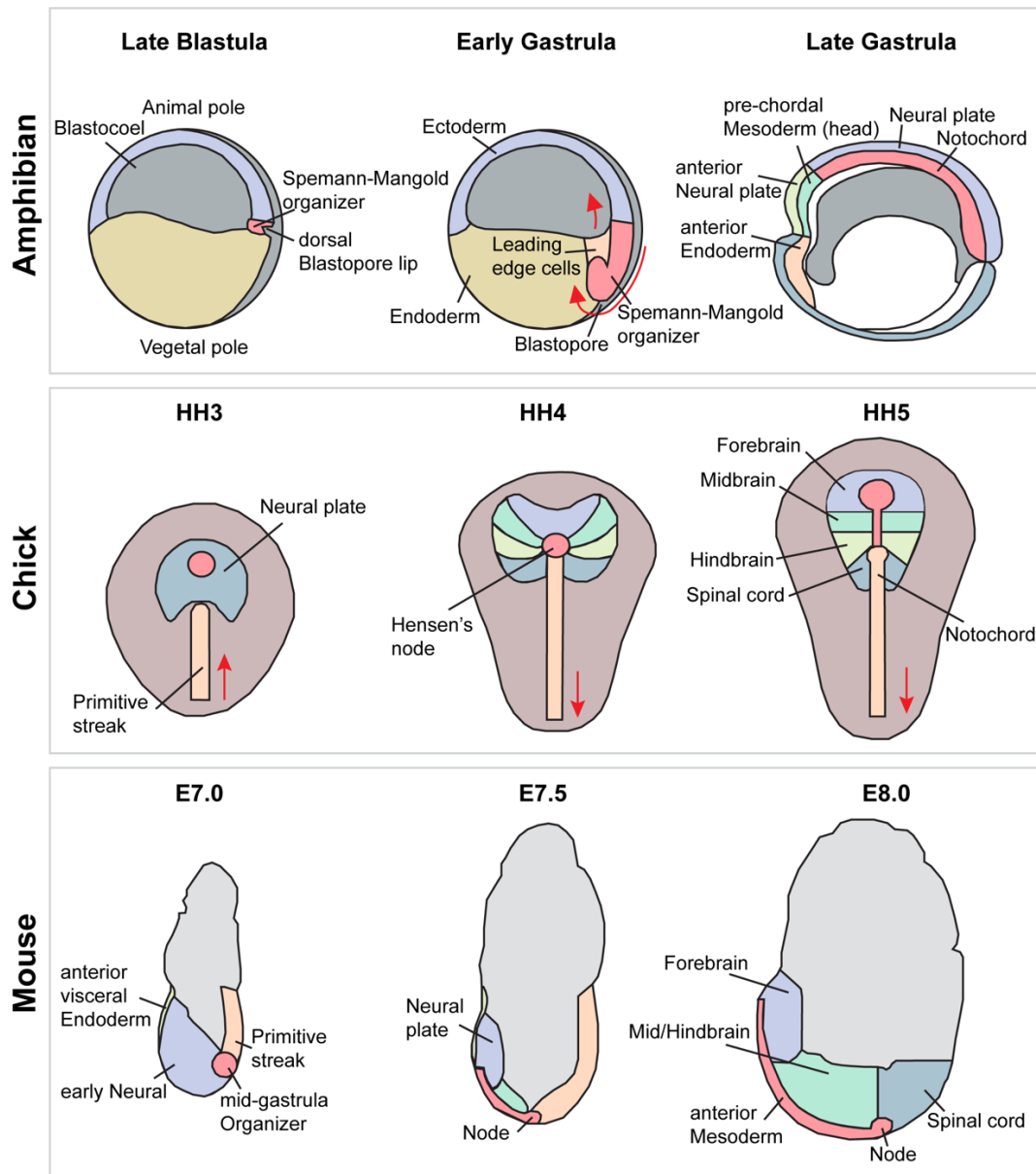


**Figure 1: Comparison of neural induction in different species. Top:** Neural induction in the amphibian embryo: In the late blastula stage, the Spemann-Mangold organizer is located at the dorsal lip of the blastopore, through which ingression occurs. During the gastrula stage, tissue further invaginates to become mesoderm (head mesoderm and notochord), and vegetal cells form endoderm, while the neural plate is formed from ectoderm. **Middle:** Neural induction in the chick: At stage HH3, the primitive streak extends anteriorly towards the neural plate and induces the formation of the Hensen's node at its distal tip. By stage HH4, the primitive streak starts to regress, and stable neural fates are established via the Hensen's node's repression of non-neural fates. By stage HH5, neural fates are determined, and the notochord has formed at the anterior end of the primitive streak, while the embryo continues to extend caudally, and the neural plate expands laterally. **Bottom:** Neural induction in the mouse: At stage E7.0, the mid-gastrula organizer, which develops into the node, forms at the distal tip of the primitive streak, and early neural tissue becomes visible. By stage E7.5, both the node and neural precursors of early neural tissue extend and migrate anteriorly. By stage E8.0, the primitive streak has disappeared, and the determination of anterior neural tissue is completed.

## I.4 Neural tube: formation and patterning

Once the neural tube is closed, it consists of various neural progenitor cells that require further signals from surrounding tissues to define their fate, leading to the differentiation of various types of neurons and glial cells (**Figure 2A**). The fate of each progenitor cell is determined by refined concentrations of morphogens secreted by ventral and dorsal signaling centers located on opposing sites of the neural tube (Jessell, 2000; L. Wilson & Maden, 2005; Ulloa & Briscoe, 2007; Ulloa & Martí, 2010; Le Dréau & Martí, 2012).

Interaction of neural ectoderm with non-neural ectoderm can induce the expression of dorsal markers as well as the formation of neural crest cells (Dickinson et al., 1995), a group of cells uniquely present in vertebrates that migrate throughout the body and form numerous derivatives (Sieber-Blum & Cohen, 1980; Baroffio et al., 1988; Bronner-Fraser & Fraser, 1988; Bronner & LeDouarin, 2012). Cell specification in the dorsal neural tube is a complex process regulated by molecules of the BMP and WNT pathways secreted by the roofplate (Liem et al., 1995, 1997; Marcelle et al., 1997), which is a signaling center located at the dorsal midline of the neural tube (Krispin, Nitzan, & Kalcheim, 2010; Krispin, Nitzan, Kassem, et al., 2010; Zagorski et al., 2017). BMP4 and BMP7, which are initially produced and secreted by the epidermal ectoderm, initiate the formation of the roof plate (Chizhikov & Millen, 2004). More recent studies have reported an involvement of Notch signaling in roofplate formation (Ofek et al., 2021). These findings highlight the complex molecular interplay between neural and non-neural ectoderm involved in the formation of neural crest cells and the specification of the dorsal neural tube through BMP, WNT, and Notch signaling pathways.

The secreted BMP and WNT molecules establish a concentration- and location-specific gradient that allow the formation of distinct domains in which a cascade of downstream transcription factors is activated to pattern dorsal neuronal cell types (**Figure 2B**) (Ulloa & Martí, 2010). The most dorsally located progenitors (dl1-dl6) give rise to different classes of interneurons, with dl1 receiving the highest concentrations of BMPs and WNTs (Gilbert, 2000). In response to BMP and WNT signaling from the roofplate and ectoderm above, the upregulated transcription factors PAX3 and PAX7 are essential for establishing distinct progenitor domains along the dorsoventral axis and the specification of dorsal interneurons, together with MSX1 and MSX2, and NGN1 and NGN2 (Liem et al., 1995, 1997).

In contrast, a gradient of the protein Sonic hedgehog (SHH) secreted by the signaling center located at the ventral midline of the neural tube, the floorplate, controls the establishment of ventral interneurons (v0-3) and motoneurons (MN) (**Figure 2B**) (Yamada et al., 1991, 1993; Marti et al., 1995; Jeong & Epstein, 2003; Chesnutt et al., 2004; Chamberlain et al., 2008). Similar to pattern induction by the roofplate, most ventrally located domains close to the floorplate receive highest concentrations of SHH (Dessaud et al., 2007), with the transcription factor NKX2.2 marking the second-most ventral domain v3 after the floorplate, and OLIG2 marking the slightly more dorsally positioned MN domain (Jessell & Dodd, 1992; Dessaud et al., 2007; Chamberlain et al., 2008). This process is mediated through the formation of an extracellular gradient of SHH coming from the floorplate, along with a graded distribution of GLI transcriptional factors within adjacent responding cells (Dessaud et al., 2007, 2008). Cells located near the floorplate, which receive higher concentrations of SHH, show also higher levels of GLI activators and reduced levels of GLI repressors. Conversely, cells located further away from the floorplate show an inverse distribution of GLI transcription factors. This differential GLI activity results in the expression of specific GLI-responsive genes within restricted domains, thereby establishing the defined ventral pattern essential for neural development.

It has been proposed that the induction of the floorplate can be initiated by inductive cues received from the underlying notochord, but, despite its highly conserved function, there seem to be differences in the mechanisms that induce floorplate cells along the anterior-posterior body axis, together with a divergence among vertebrate species. In addition, also other signaling molecules such as RA secreted from the adjacent somites have been reported to be involved in dorsoventral patterning of the neural tube (Ulloa & Martí, 2010).

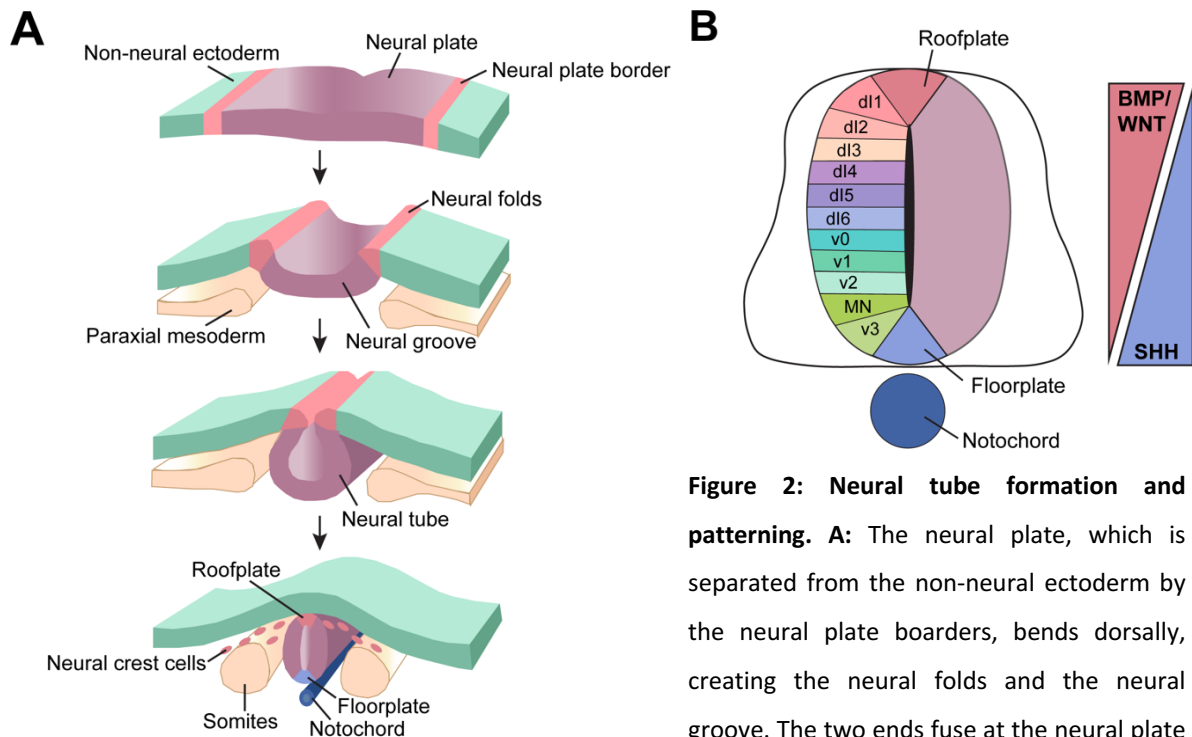


**Figure 2: Neural tube formation and patterning.** **A:** The neural plate, which is separated from the non-neural ectoderm by the neural plate borders, bends dorsally, creating the neural folds and the neural groove. The two ends fuse at the neural plate borders, resulting in the closure of the neural

tube. Neural crest cells emerge from the dorsal side of the neural tube, and two opposing signaling centers, the roofplate and floorplate, are established. The notochord forms underneath the neural tube from mesoderm after gastrulation is completed. Paraxial mesoderm differentiates to form the somites. **B:** Progenitor domains of specific neuronal subtypes (v0-v3, MN, dl1-dl6) are distributed along the dorsoventral axis of the neural tube. This pattern of gene expression is progressively established in response to opposing morphogen gradients secreted from the ventral floorplate and notochord (SHH, blue), and from the dorsal roofplate (BMP and WNT, brick red).

## 1.5 Organizers in (ventral) neural tube development

The induction of neural tissue in the developing embryo, the formation of signaling centers in the neural tube, and the establishment of correct dorsoventral patterning within the neural tube all rely on defined organizers. These organizers play a crucial role in directing embryonic development through specific inductive signaling mechanisms. Regulatory organizers represent specific areas or groups of cells within the early vertebrate embryo that induce directed cell differentiation and generate patterns to ensure the correct formation of organized tissues along the body axis, which are essential for proper embryonic development (Spemann & Mangold, 1924; Gurdon, 1987; Gerhart, 2001; C. Anderson & Stern, 2016). Organizers fulfill their function through morphogenic signaling, in which one tissue secretes

signals at a specific time that influence another part of the embryo, leading to the upregulation of a gene regulatory network sufficient to induce formation of new structures that would otherwise not develop. Only a few organizers have been identified and described so far, due to the complexity of the vertebrate embryo (Kiecker & Lumsden, 2012).

In the early 1920s, first described by Hans Spemann and Hilde Mangold, the Spemann-Mangold organizer was identified as the main organizer in amphibians (Spemann & Mangold, 1924). By doing grafting experiments using two newt species, the researchers identified a specific region on the dorsal lip of the blastopore of the embryo that has the ability to induce the formation of a secondary body axis with neural tissue including a second neural tube, notochord and somites in adjacent ectodermal cells (R. Harland & Gerhart, 1997; Stern, 2001; Gerhart, 2001). This was the first demonstration that the fate of cells can be influenced and changed by surrounding cell populations.

Among the organizers involved in the development of the nervous system are the Spemann-Mangold organizer (similar to the node in mammalian species/ Hensen's node in avian species), which represents a primary organizer due to its crucial role in neural induction; and secondary organizers such as the notochord and floorplate for dorsoventral pattern induction, and the mid-hindbrain boundary (Isthmic organizer) for anterior-posterior pattern induction (Vieira et al., 2010). Primary organizers have the ability to form complete additional body axes including different types of neural, epidermal and mesodermal tissue. In contrast, secondary organizers form as specialized parts of the neuroepithelium that secrete morphogens to pattern surrounding tissue (Kiecker & Lumsden, 2012). The difference between primary and secondary organizers are that secondary organizers can induce different fates based on varying cellular and environmental context, therefore often act as molecular boundaries that prevent mixing of different cell types (Vieira et al., 2010; Kiecker & Lumsden, 2012). When the secondary organizers notochord and floorplate were grafted onto the limb bud, a structure of undifferentiated mesodermal cells that will form a fully patterned limb, these tissues were able to induce axis duplication but of limb and digit patterns in mouse and chick embryos rather than inducing ventral neural tube patterning (Hornbruch & Wolpert, 1986; B. L. M. Hogan et al., 1992; Wagner et al., 1992), due to their

ability to secrete RA and SHH signaling, providing evidence that these tissues have organizer properties.

It is commonly accepted that the Spemann-Mangold organizer, node, and Hensen's node can induce a broad variety of cell fates and tissue structures due to their relatively pluripotent cellular nature, and their permissive, not yet patterned environment. As development progresses, other organizers, such as the notochord and floorplate, induce specific patterns based on the morphogens they secrete and their already primed environment. Therefore, if these secondary organizers are transplanted to a limb, they are more likely to give rise to limb-specific tissue rather than neural tissue (Hornbruch & Wolpert, 1986). To conclude, understanding the activity of an organizer is not only based on its actions, but rather on the competence state of the responsive tissue (Martinez Arias & Steventon, 2018).

While there are studies indicating that the Spemann-Mangold organizer and the node fulfill similar functions during neural induction, some debates persist on this topic and if they can indeed be called 'alike'. While the Spemann-Mangold organizer is able to give rise to a complete secondary body axis containing notochord, neural tube and somites when transplanted to another amphibian embryo, transplantation assays of node tissue performed between two mouse embryos of the same developmental stage result in a secondary body axis but only notochord and somites were derived from the donor, while the neural tube was formed by cells from the host (Beddington, 1994). It has been suggested that this discrepancy could result from a lower starting cell number in the mouse embryo respectively organizer present relative to other species such as amphibian or chick embryos (Snow, 1977; Lawson & Pedersen, 1992; J. L. Smith et al., 1994; Martinez Arias & Steventon, 2018). However, it was shown that node tissue from mouse embryos can induce a complete neural axis including forebrain structures upon grafting to chick embryos (Kintner & Dodd, 1991; Knoetgen et al., 2000).

In this dissertation, I focus on three organizers that are crucial for the development of the CNS in vertebrate embryos: the node, notochord and floorplate.

### I.5.1 Node

One of the most important organizers during embryonic development, that orchestrates neural induction and patterning of the mammalian embryo and various other species is the node (Hamburger, 1988; Zhou et al., 1993; Beddington, 1994; R. Harland & Gerhart, 1997; S.-L. Ang & Behringer, 2002). The node forms as a result of gastrulation at the anterior end of the primitive streak at the distal tip of the mouse embryo (Sulik et al., 1994). In mouse developmental stages, this occurs at approximately E7.5 (Downs & Davies, 1993). The node is essential for the establishment of the body axes, the formation of somitic mesoderm, neural tissues and the notochord (P. P. L. Tam & Behringer, 1997; Balmer et al., 2016; Solovieva et al., 2022). Due to its ability to form and organize a secondary body axis when transplanted to the lateral side of a host embryo, the node has been suggested to be the equivalent of the amphibian Spemann-Mangold organizer, although there is some debate (Spemann & Mangold, 1924; Waddington, 1932; Sulik et al., 1994; Beddington, 1994; Streit et al., 1998). In avian species such as chick and quail, the node is commonly referred to as the Hensen's node (Joubin & Stern, 1999; Selleck & Stern, 1991), which forms at the beginning of gastrulation approximately 20 hours after fertilization referring to Hamburger and Hamilton stage 3-4 (HH3-4) (Hamburger & Hamilton, 1992).

As described in “I.3 Neural induction”, the node secretes the signaling molecules Noggin (Lamb et al., 1993; W. C. Smith & Harland, 1992), Chordin (Piccolo et al., 1996; Sasai et al., 1995), Follistatin (Hemmati-Brivanlou et al., 1994), Nodal (Zhou et al., 1993) and others, thereby promoting the formation of neural tissue from the ectoderm by inhibiting BMP signaling. This proposes that in the absence of BMP signaling, ectodermal cells will become neural tissue (Muñoz-Sanjuán & Brivanlou, 2002). It has been further suggested that the node and the anterior primitive streak, where the node derives from, are direct sources of these BMP inhibitors and neural-inducing factors, due to the expression of the early midbrain marker *Engrailed* (Hemmati-Brivanlou & Harland, 1989; Klingensmith et al., 1999). However, cell grafting experiments performed in early chick embryos reported that BMP inhibition through the expression of Noggin or Chordin was not sufficient to induce neural tissue from non-neural cells, though probably caused by an incomplete inhibition of all BMPs present in the ectoderm (Streit et al., 1998; Muñoz-Sanjuán & Brivanlou, 2002).

In addition to prominent expression of BMP inhibitors to induce neural ectoderm formation, the node also produces SHH, which is crucial for the proper differentiation and development of ventral neural tube tissues (Martí, Takada, et al., 1995; Charrier et al., 1999; Jeong & Epstein, 2003). Studies performed in chick embryos and chick-quail chimeras have revealed regional differences in SHH expression within the node (Charrier et al., 1999). SHH is strongly expressed on the rostral side of Hensen's node, both dorsally and ventrally, which will eventually differentiate into the floorplate and notochord. In contrast, in the caudal node, SHH expression is less abundant and primarily localized in ventral cells. In the mouse node, *Shh* enhancer activity was detected in regions from which both the floorplate and notochord will derive from (Jeong & Epstein, 2003), further highlighting the importance of SHH expression from this organizer. However, different *Shh* reporter expression in mouse embryos suggests that the floorplate and notochord may originate from distinct precursor cells within the node.

Furthermore, scientists have shown that the Hensen's node plays a crucial role in anterior-posterior axis patterning during avian development by secreting RA that can induce posteriorization, but not anteriorization of the embryo (B. L. M. Hogan et al., 1992; Y. Chen et al., 1992; Itasaki et al., 1996; Grapin-Botton et al., 1997; Muhr et al., 1999; Maden, 1999; Liu et al., 2001; Bel-Vialar et al., 2002). Similarly, also the node in mouse embryos has been suggested to be a source of RA (H. L. Ang et al., 1996; Niederreither et al., 1999).

The transcription factor FOXA2 (or also known as *HNF-3 $\beta$* ) is essential for node and notochord formation thus proper development of the mouse embryo (S.-L. Ang & Rossant, 1994; Weinstein et al., 1994). *FoxA2* mutant mouse embryos lack a morphological node and its derivatives but still undergo neural induction (Klingensmith et al., 1999), suggesting that there could be additional mechanisms involved and indicating that maintaining an organizer-like signaling center is not necessary for neural induction. When ablating node cells during the late mouse gastrulation, the resulting embryos showed intact though shortened anterior-posterior body axes with typical head fold, neural tube, and primitive streak morphology and only slightly delayed somite formation (B. P. Davidson et al., 1999; B. P. Davidson & Tam, 2000). In this study, the researchers reported a partial restoration of the neural tube and

paraxial mesoderm due to recruitment of cells from neural ectoderm and somitic mesoderm in surrounding tissues.

While the structures that have been induced and organized by the node continue to develop and differentiate, the node itself is a transient structure that disappears as development progresses (Jurand, 1974; Sulik et al., 1994; Corallo et al., 2015). The notochord, whose formation is induced by the node, continues to play a significant role in the development of the axial skeleton and the nervous system (Placzek, 1995; B. P. Davidson et al., 1999; B. P. Davidson & Tam, 2000).

### 1.5.2 Notochord

The notochord is a direct derivative of the node that plays a fundamental role in the development of vertebrate embryos by serving as a primary regulatory organizer of the neural tube that induces the formation of floorplate cells via inductive cues and SHH signaling. During gastrulation, the notochord forms by anteriorly migrating mesodermal cells as an extension of the node (Sulik et al., 1994; Stemple, 2005). After aggregation of these mesodermal cells, they transform and condense into the typical rod-like structure with a fluid-filled lumen, the so-called notochordal canal, directly underneath the ventral midline of the developing neural tube. It extends from the cranial to the caudal end along the axial midline of the developing vertebrate embryo (Jurand, 1974; P. P. Tam & Beddington, 1987; Sulik et al., 1994). However, more recent studies propose an alternative mechanism for notochord formation, suggesting that it may originate at cervical levels of the embryo and subsequently elongate in both cranial and caudal directions via anterior expansion and posterior addition, with the most anterior region supposed to form from a different cellular origin (Yamanaka et al., 2007; De Bree et al., 2018; McLaren & Steventon, 2021). The notochord plays a critical role in patterning surrounding tissues, including the ventral neural tube and somites, through the secretion of the signaling molecule SHH to adjacent regions (Martí, Takada, et al., 1995). Additionally, it functions as axon guidance to cross the midline during neurogenesis (Placzek et al., 1990; van Straaten et al., 1985; Yamada et al., 1991).

It has been shown that the notochord is sufficient to induce the formation of the floorplate through secreting the morphogen SHH (Placzek et al., 1990). By grafting an ectopic notochord underneath the lateral neural plate and neural tube in chick embryos, additional floorplate formation was observed (van Straaten et al., 1985), whereas upon removal of the notochord the neural tube developed without a floorplate and motoneurons, providing evidence that local signal from the notochord can induce functional floorplate (Placzek et al., 1990). In zebrafish embryos, floorplate cells persist despite the ablation of notochordal cells, suggesting the notochord is not needed for floorplate formation in this species (Shih & Fraser, 1995). Instead, it was shown that the notochord can induce so-called slow muscle formation from medial somite-derived cells in zebrafish embryos via the secretion of SHH (Blagden et al., 1997), indicating its broad role as an organizer.

### 1.5.3 Floorplate

Due to its central role in ventral neural tube patterning, the floorplate is considered a crucial organizer of nervous system development. Located at the ventral midline of the neural tube, it secretes the signaling molecule SHH, which acts as a morphogen to regulate cell differentiation and patterning along the ventral-to-dorsal axis of the neural tube, thereby directing the specification of different neuronal cell types (Martí, Takada, et al., 1995; Ribes et al., 2010). Besides SHH, the floorplate is marked by the expression of FOXA2, ARX and Netrin-1 (Ribes et al., 2010; Cho et al., 2014; Dominici et al., 2017). The floorplate possesses a wedge-shaped structure and is divided into medial and lateral floorplate based on the spatial expression pattern of certain proteins: while SHH expression is restricted to medial floorplate cells, FOXA2 protein is found in a broader area of the ventral neural tube (Sasaki & Hogan, 1993; Monaghan et al., 1993; S.-L. Ang & Rossant, 1994; Roelink et al., 1994; Schauerte et al., 1998; Schäfer et al., 2005, 2007).

The importance of FOXA2 in the development of axial structures in vertebrates was highlighted by studies showing that mouse embryos lacking this gene neither develop a notochord nor the floorplate (S.-L. Ang & Rossant, 1994; Weinstein et al., 1994). Thus showing that, among its numerous functions, FOXA2 is necessary for node, notochord and floorplate formation (Ruiz i Altaba et al., 1993; Tamplin et al., 2008). Furthermore, it is one of the earliest

markers that distinctly identifies midline cells on the surface of the developing embryo before they form the notochord (Sasaki & Hogan, 1993; Bartscher & Lickert, 2009).

Numerous studies have reported fundamental differences in floorplate induction between species, and also within the same species along the anterior-posterior body axis, suggesting that floorplate cells might differ in their origin and identity (**Figure 3**) (Le Douarin & Halpern, 2000; Placzek et al., 2000; Patten et al., 2003; Kremnyov et al., 2018). Probably the most commonly accepted mechanism for floorplate formation in vertebrates is via the induction of the notochord through SHH signaling to the adjacent neural tube (van Straaten et al., 1985; Placzek et al., 1990; Yamada et al., 1993; Jeong & Epstein, 2003). FOXA2 expression is subsequently activated in cells destined to become the floorplate, which then subsequently also expresses SHH (Roelink et al., 1994, 1995). Gain-of-function experiments have demonstrated that SHH can induce the ectopic differentiation of floorplate cells in the neural plate *in vitro* (Roelink et al., 1994, 1995; Martí, Bumcrot, et al., 1995). Furthermore, inducing SHH expression in the notochord in mouse and rat embryos lead to its activation in the neural tube (Echelard et al., 1993; Roelink et al., 1994; Chamberlain et al., 2008). Conversely, blocking SHH signaling in the notochord abolished its ability to induce floorplate cells, as shown in studies by Ericson et al. (Ericson et al., 1996), confirming its sufficiency in floorplate induction.

However, observations of clustered FOXA2 expression along the ventral midline and node prior to notochord formation during the headfold stage of mouse embryos (Sasaki & Hogan, 1993, 1994; Balmer et al., 2016), combined with its extensive expression in various ventral tissues such as the endoderm, node, notochord, and floorplate, question that FOXA2 expression is only a result of the inductive influence of the notochord (Sasaki & Hogan, 1996; Teillet et al., 1998; Balmer et al., 2016). In addition, several studies performed in chick embryos and chick-quail chimeras have also questioned this model of notochord-dependent floorplate induction (van Straaten & Hekking, 1991; Patten et al., 2003). Instead researchers suggest that, especially in avian species, anterior floorplate cells are derived by a distinct population of pre-specified cells from the node (I Altaba et al., 1995; Catala et al., 1996; Teillet et al., 1998; Le Douarin & Halpern, 2000; Patten et al., 2003), while only posterior floorplate cells could need inductive cues from the notochord (Patten et al., 2003).

Other experiments performed in zebrafish mutants and chick-quail chimeras indicate a dual origin of floorplate formation. These studies suggest that the medial floorplate forms from the node and expresses SHH, which subsequently induces adjacent lateral floorplate cells in the neural tube (Schäfer et al., 2005, 2007). In zebrafish embryos, while SHH signaling is necessary for the maintenance of the medial floorplate, its initial induction does not rely on SHH (Shih & Fraser, 1995), but rather depends on Nodal signaling (Schauerte et al., 1998; Odenthal et al., 2000; W. Chen et al., 2001; Etheridge et al., 2001; Varga et al., 2001).

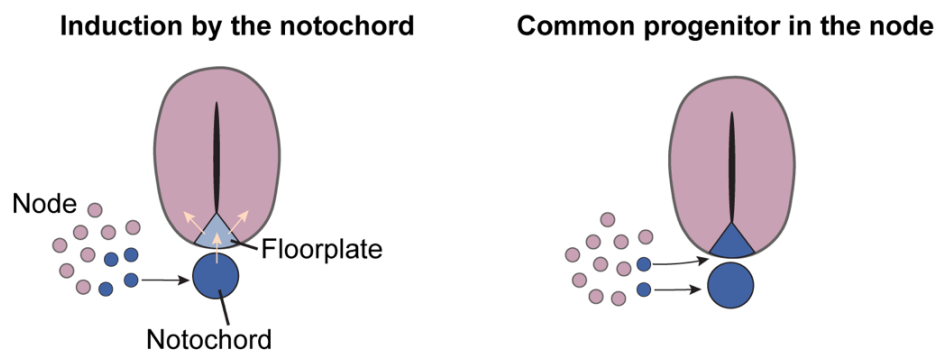


**Figure 3: Models of floorplate induction.** **Left:** Node-derived notochord (dark blue) locally induces the formation of a floorplate (light blue) in the adjacent neural tube via SHH signaling (peach arrow). In turn, the floorplate becomes an organizer itself and patterns the neural tube through SHH signaling (peach arrows). **Right:** Both the notochord and the floorplate originate from common progenitor cells located in the node.

## I.6 Other factors contributing to floorplate formation/ ventral patterning

As already described above, SHH signaling from the node, notochord and ventral floorplate is commonly accepted as a crucial mechanism for inducing pattern formation, leading to the specification of various ventral neural cell types essential for proper nervous system development (Martí, Takada, et al., 1995; Martí, 2002; Ribes et al., 2010).

While the critical role of notochordal-derived SHH in vertebrate floorplate formation and floorplate-derived SHH in ventral neural tube patterning has been extensively studied, it is important to consider also other significant factors and signaling pathways that may contribute to floorplate formation and ventral patterning. Several studies have reported controversial results regarding the necessity of SHH from the notochord in floorplate induction, with some even suggesting that SHH might be dispensable for initial induction in

defined anterior-posterior locations and in certain species (Shih & Fraser, 1995; Patten et al., 2003). This implies that the initial induction of the floorplate, especially in most anterior regions, could possibly rely on other signals or a combination of multiple factors, and SHH primarily being required for dorsoventral patterning.

### I.6.1 RA signaling

Initially, RA is generated in the paraxial mesoderm by specific dehydrogenase enzymes produced in the notochord (RALDH2, frog), and in the primitive streak (Cyp1B1, chick) (Swindell et al., 1999; Diez del Corral et al., 2003; L. Wilson & Maden, 2005). Later in development, when somites have differentiated, they also begin to express RALDH2 which subsequently synthesizes RA (Maden et al., 1998).

Also, the Hensen's node in chick and the node in mouse embryos is suggested to secrete RA to posteriorize the embryo during development (B. L. M. Hogan et al., 1992; Y. Chen et al., 1992; Itasaki et al., 1996; H. L. Ang et al., 1996; Grapin-Botton et al., 1997; Muhr et al., 1999; Maden, 1999; Niederreither et al., 1999; Liu et al., 2001; Bel-Vialar et al., 2002). Additionally, studies of grafting node and floorplate cells from chick and mouse embryos to limb bud further provided evidence, that these tissues represent a source of RA (Hornbruch & Wolpert, 1986; B. L. M. Hogan et al., 1992; Wagner et al., 1992), due to their effect of generating digit and/ or limb duplications similar to what have been observed when locally applying RA to limb bud (Tickle et al., 1982).

RA signaling is involved in the induction of axial patterning, neural differentiation, motor axon outgrowth, and neural patterning during development of various vertebrate species (Maden, 1996, 2007; Duester, 2008). It is essential for patterning both the anterior-posterior and dorsoventral axes of the neural plate and neural tube. In the anterior-posterior axis, RA acts together with WNTs and FGFs to specifically organize the development of the posterior hindbrain and anterior spinal cord. This coordinated activity ensures a proper formation and differentiation of these critical neural regions (Liu et al., 2001; Maden, 1996, 2002, 2007; Melton et al., 2004), while in the absence of RA signaling chick embryos lack large parts of their posterior hindbrain and the anterior spinal cord appears abnormal (Maden, 1996;

Maden et al., 1996; L. Wilson et al., 2004). Additionally, more recent studies suggest that RA is also involved in the patterning of the forebrain in mouse embryos (Haushalter et al., 2017).

Vitamin A-deficient (VAD) quail embryos, which lack RA signaling, show a disruption of genes important for induction and specification of dorsoventral progenitor populations, causing an uneven dorsal expansion of ventral-specific genes and a reduction in the expression of roofplate and dorsal patterning genes in general (L. Wilson et al., 2004; L. Wilson & Maden, 2005). Inversely, RA-dependent inhibition of BMP signaling leads to complete neural crest cell production and emigration (Rekler & Kalcheim, 2022). This suggests that, together with SHH and BMP proteins, RA is essential for the dorsoventral patterning of the neural tube (Sasai & De Robertis, 1997; Novitsch et al., 2003; Chambers et al., 2007; Ribes et al., 2009).

### 1.6.2 BMP signaling

Extracellular antagonists of BMPs, such as Noggin or Chordin, play significant roles in neural induction during embryonic development. Also, as described above, BMP signaling plays a crucial role in formation of the dorsal signaling center, the roofplate, as well as in neural crest cell and neural cell specification of the dorsal neural tube. However, there is evidence in the literature indicating that BMP signaling also plays a role in the patterning of the ventral neural tube, despite this information often being overlooked.

BMPs are members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily that signal via type I and type II serine/threonine kinase receptors and intracellular Smad transcription factors (Kingsley, 1994; B. L. Hogan, 1996; G. J. Anderson & Darshan, 2008; Todd et al., 2020). Initially acknowledged for their role in bone regeneration, BMPs serve as useful regulators in development and tissue homeostasis (Kingsley, 1994; Nakase et al., 1994; B. L. Hogan, 1996). They function by binding to complexes of two BMPR-I and two BMPR-II receptors, modulating the expression of target genes via various signal transduction pathways. Among these, the SMAD signaling pathway has been extensively studied. Following binding of BMP ligand dimers like BMP2, 4, or 7 to their receptors, downstream SMAD1/5/9 undergo phosphorylation, then form complexes with SMAD4, and subsequently translocate into the nucleus where they induce the expression of specific target genes. The mitogen-activated

protein (MAP) kinase pathway and possibly other pathways could potentially also be activated through BMP ligand binding (Yamaguchi et al., 1995; Derynck & Zhang, 2003).

BMP activity can be inhibited through various mechanisms, such as the expression of dominant negative non-signaling pseudo receptors like BAMBI (Onichtchouk et al., 1999), the blocking of BMP signaling by inhibitory SMADs 6 and 7 (Hayashi et al., 1997; Nakao et al., 1997; Hata et al., 1998; Bai et al., 2000), the interference of intracellular SMAD binding proteins, or through extracellular antagonist BMP binding proteins including Chordin, Noggin, and Follistatin (Piccolo et al., 1996; Zimmerman et al., 1996; Blader et al., 1997; Fainsod et al., 1997; Canalis et al., 2003; Yanagita, 2005).

BMP pathway members have been reported to be expressed in the ventral neural tube *in vivo*, suggesting a potential role in floorplate formation and ventral patterning. It is known that the notochord expresses BMP inhibitors such as Noggin and Chordin (K. Dale et al., 1999; McMahon et al., 1998; Stottmann et al., 2006). Even further, in mouse mutants deficient in one or both of the mentioned inhibitors, BMP4 was found ectopically expressed in the notochord and ventral neural tube (McMahon et al., 1998). Additionally, also other studies reported expression and involvement of BMP proteins in ventral neural tube patterning (K. Dale et al., 1999; Furuta et al., 1997). These observations could possibly suggest that the ventral domains of the neural tube can exhibit characteristics typically associated with dorsal signals (Furuta et al., 1997; McMahon et al., 1998; K. Dale et al., 1999), but are suppressed by the local presence of BMP inhibitors and ventralizing signals. In the chick embryo, BMP ligands and the BMP inhibitor Noggin can be detected in the Hensen's node as well as notochord (Faure et al., 2002) and floorplate, suggesting a dynamic pattern of BMP (K. Dale et al., 1999; Vesque et al., 2000). It might be possible that BMP signaling in the notochord is activated locally by ligands that are resistant to BMP inhibitors, or alternatively that a locally expressed antagonist of BMP inhibitors, such as a Tolloid-like protease in the chick, may enable BMP signaling in the notochord (Liaubet et al., 2000; Martí, 2000; Piccolo et al., 1997). These findings suggest that the spatial regulation of BMP signaling, determined by BMP ligands and their inhibitors, plays a crucial role in regulating ventral neural tube patterning across species.

### I.6.3 YAP signaling

Very recently, scientists have reported a structural role for notochord-dependent induction of the floorplate in the mouse due to mechanisms other than secreted SHH, namely due to mechanotransduction via Yes-associated protein 1 (YAP1) (Cheng et al., 2023). YAP signaling, respectively the Hippo pathway, regulate multiple developmental processes, such as cell proliferation, migration, survival, and differentiation (Kumar et al., 2019). YAP and its ortholog, transcriptional coactivator with a PDZ-binding domain (TAZ), function as transcriptional coactivators and serve as effector proteins in the Hippo pathway. This pathway, a serine/threonine kinase module, phosphorylates YAP on specific serine residues, resulting in its sequestration in the cytoplasm and consequently limiting its transcriptional coactivation activity.

While it is known that FOXA2 and SHH are regulators of notochord and dorsoventral pattern formation in the neural tube, the mechanisms underlying this region-specific expression of FOXA2 and SHH have remained unknown. Interestingly, activation of *Yap1* by a gradient of mechanical stress and tissue stiffness in the notochord and neural tube lead to induction of *FoxA2* and *Shh* at the highest mechanical stress (Cheng et al., 2023). Moreover, it has been demonstrated that YAP signaling is both necessary and sufficient for controlling ventral-to-dorsal patterning. While activation of SHH signaling can compensate for neural tube patterning defects resulting from the absence of YAP, it is not sufficient to restore notochord formation. This indicates that mechanotransduction via YAP functions by activating FOXA2 expression in the notochord and at the same time interacts with FOXA2 to induce SHH expression in the floorplate. These findings reveal a crucial and novel role of YAP in integrating mechanical stimuli with biochemical signaling activation to establish signaling centers important for embryonic development and patterning.

It further has been reported that YAP signaling plays a crucial role in ventral progenitor formation in the developing spinal cord of mouse and chick embryos due to control of YAP activity by an atypical cadherin called FAT3 (Seo et al., 2022).

Besides the important functions of YAP signaling in controlling ventral neural tube fates, it has been reported that YAP activity together with BMP and WNT signaling is also important for

dorsal neural tube specification and neural crest emigration in quail and chick embryos (Kumar et al., 2019).

## 1.7 Self-organization: a mechanism to organize the organizer?

While organizers play a crucial role in directing embryonic development through specific inductive signaling mechanisms, self-organization in biology demonstrates how complex patterns and structures can emerge autonomously from local or distant interactions between cells. Remarkably, recent studies have demonstrated that organizers can form *in vitro* through self-organization, even in the absence of their embryonic inducers (Sato et al., 2009; Eiraku et al., 2011; A. Meinhardt et al., 2014; Martyn et al., 2018).

Self-organization is defined as something to spontaneously create structure and order in time and space by the interaction of its individual 'unstructured' components, and mostly without specific external instructions. This phenomenon is driven by internal interactions and dynamic processes, leading to complex organization and functionality of various tissues. Several mechanisms have been proposed to account for self-organization and the formation of complex patterns and specific structures. These mechanisms can operate over long distances involving morphogen signaling, or act over short distances through interactions mediated by direct cell-cell and cell-environment contacts.

In order to be able to self-organize to create a pattern, very often the initial symmetry has to be broken in the first place. In that case, the starting point is homogenous or as stated by the Nobel Laureate in Physics P.W. Anderson: "*the existence of different viewpoints from which the system appears the same*" (P. W. Anderson, 1972). Like a homogeneous mass of cells in the zygote that continues to divide until it finally breaks symmetry to form different cell types. These cell types segregate to form a cluster of pluripotent stem cells (PSCs) from the inner cell mass (ICM), which has the potential to form all cells in the organism, and the trophoblast, which will become extraembryonic tissue.

Various mechanisms have been proposed to explain how self-organization and (periodic) pattern formation occurs during the development of vertebrates (Hiscock & Megason, 2015).

In this thesis the following mechanisms that act over long ranges and involve morphogen signaling are discussed: 'Reaction-Diffusion' by Alan Turing (Turing, 1952), 'Local Autoactivation-Lateral Inhibition' by Alfred Gierer and Hans Meinhardt (Gierer & Meinhardt, 1972), and 'Positional Information' by Lewis Wolpert (Wolpert, 1969) (**Figure 4**). Additionally, this thesis examines the following mechanisms operating over short ranges through direct cell-environment interactions and cell-cell contacts: collective cell migration, sorting and mechanochemical feedback loops (**Figure 5**).

## I.8 Long-range mechanisms

### I.8.1 'Reaction-Diffusion' by A. Turing

"The Chemical Basis of Morphogenesis" by A.M. Turing laid the foundation for understanding how complex biological, self-organized patterns and structures emerge in development from initially uniform conditions through a process known as reaction-diffusion (Turing, 1952). He proposed that the interaction of chemical substances, so-called morphogens, could lead to the formation of stable spatial patterns via a reaction-diffusion system, where morphogens react in a certain manner that promote the production of one morphogen while inhibiting the production of another, and that these molecules diffuse through the tissue at different rates (**Figure 4A**). The theory is based on short range activation and long range inhibition, leading to formation of periodic patterns from a homogeneous population creating positional information *de novo* (H. Meinhardt & Gierer, 2000). Reaction-diffusion patterns are sensitive to external perturbations and have the remarkable ability to regenerate after such perturbations (Gierer & Meinhardt, 1972; Kondo, 2017). This model is also often referred to as the activator-inhibitor model. Initial instability or molecular fluctuations of the activator lead to its expression. The activator then promotes its own expression and that of an inhibitor, which slows down the activator's expression. The inhibitor is meant to diffuse faster than the activator, causing the activator's suppression between its peaks of expression, which creates a periodic pattern. In contrast, the substrate-depletion model, which is also a type of reaction-diffusion, involves an activator that consumes a substrate, creating localized depletion zones and resulting in more localized patterning. However, both models are fundamental in explaining how complex biological patterns such as spots, stripes and waves in various tissues can develop. Patterns on snail and sea shells are one of the classical examples of Turing

patterns in nature, as well as hair follicle formation in mice (Sick et al., 2006), feathers in chicks (D. Davidson, 1983; Jung et al., 1998; Jiang et al., 1999), wing patterns in butterflies (Murray, 1981), and teeth-shaped shark scales (Cooper et al., 2018).

A minimal reaction-diffusion system consists of at least two components, however, there can be many combinations using multiple components, such as: WNT-DKK in mouse hair follicle spacing (Sick et al., 2006), NODAL-LEFTY in zebrafish embryogenesis (Müller et al., 2012), BMP-SOX9-WNT in mouse digit patterning (Raspopovic et al., 2014), or GDF5-NOGGIN-pSMAD in digit segmentation patterning in birds and mammals (Grall et al., 2024)

### I.8.2 ‘Local Autoactivation-Lateral Inhibition’ by A. Gierer and H. Meinhardt

Similar to the reaction-diffusion, described by Turing, is the model of local auto-activation and lateral inhibition by A. Gierer and H. Meinhardt based on the interaction of an activator and an inhibitor. The model explains how the concentration of a short-range autocatalytic substance, the activator, controls the creation of its long-range opponent, the inhibitor (Gierer & Meinhardt, 1972; H. Meinhardt, 1996). This spatial organization of activator and inhibitor creates a balance where high concentrations of the activator are maintained in small, localized regions, while surrounding areas get suppressed due to the long-range influence of the inhibitor (**Figure 4B**). Local autoactivation ensures strong, localized signaling in certain regions, whereas lateral inhibition prevents the signal from spreading too far, thereby generating distinct patterns. While both models, Turing’s as well as Gierer-Meinhardt’s, aim to explain biological pattern formation through chemical interactions, Turing's reaction-diffusion model focuses on the differential diffusion rates of activators and inhibitors to generate stable patterns, whereas the Gierer-Meinhardt model emphasizes the balance of local autoactivation and lateral inhibition to produce localized peaks of activity, which is often used to explain more localized patterning phenomena, such as the distribution of specific cell types like the formation of sensory bristles in the adult peripheral nervous system of the fruit fly (Simpson, 1990), or localized pigmentation patterns like stripe-formation in zebrafish and marine angelfish (Kondo & Asai, 1995; Hamada et al., 2014). In a cellular context, this means that cells exhibit direct contacts between other cells (short-range) while long-range protrusion-mediated contacts cause repulsion. Under these conditions, lateral inhibition can

be mediated by the Delta-Notch signaling pathway (Collier et al., 1996; Guisoni et al., 2017). The coupling of growth with the formation of new stripes is consistent with a lateral inhibition mechanism involving the Delta-Notch signaling pathway, characterized by a fixed inhibitory distance. In this context, a cell expressing Delta ligands activates Notch receptors on neighboring cells, which in turn inhibits these neighboring cells from adopting the same fate. As a result, a pattern of alternating cell fates is established. As tissue grows, the distance between cells expressing high levels of Delta and those expressing high levels of NOTCH increases, causing the local level of inhibition to decline with distance. When the inhibitor level falls below a certain threshold due to the increased spacing from tissue growth, new cells can differentiate into a new stripe pattern. According to this model, inhibition of growth would prevent the formation of additional stripes, maintaining the existing pattern. Lateral inhibition has been extensively studied in flies, worms and vertebrates (Haddon et al., 1998).

### 1.8.3 'Positional Information' by L. Wolpert

The concept of positional information proposed by L. Wolpert explains how cells in a developing embryo use spatial cues, primarily morphogen gradients, to determine their position and differentiate into appropriate cell types (Wolpert, 1969, 1989, 2016). Wolpert illustrated this with his so-called 'French-Flag' model, showing how a single morphogen gradient could create three distinct developmental zones, analogous to the colors of the French flag (**Figure 4C**) (Wolpert et al., 2019). Morphogen gradient formation involves the localized production, diffusion, and degradation of morphogens, with these processes determining the gradient's shape and range. Cells sense and respond to morphogen concentrations via receptors and signaling pathways, leading to changes in gene expression and the activation of developmental pathways specific to their positions (Crick, 1970). This concept is demonstrated by experiments that alter morphogen levels and observe subsequent changes in cell fate. An example where positional information underlies self-organization is in the development of the chick wing, where graded, secreted SHH from a polarizing region promotes growth of the digit-forming field and specifies cells with their positional values (Pickering & Towers, 2016). Other examples are the Bicoid morphogen gradient that organizes anterior development in *Drosophila* embryos (Driever & Nüsslein-Volhard, 1988), or the dorsoventral patterning of different neural cell types by the secretion

of SHH and BMP from the opposing signaling centers in the floor- and roofplate of the neural tube (Zagorski et al., 2017).

Although positional information is not the only component of a self-organizing system, it plays an important role in facilitating self-organization during development. Interestingly, some researchers suggest an interplay between positional information and reaction-diffusion, each bringing its own advantages to the system (Green & Sharpe, 2015).

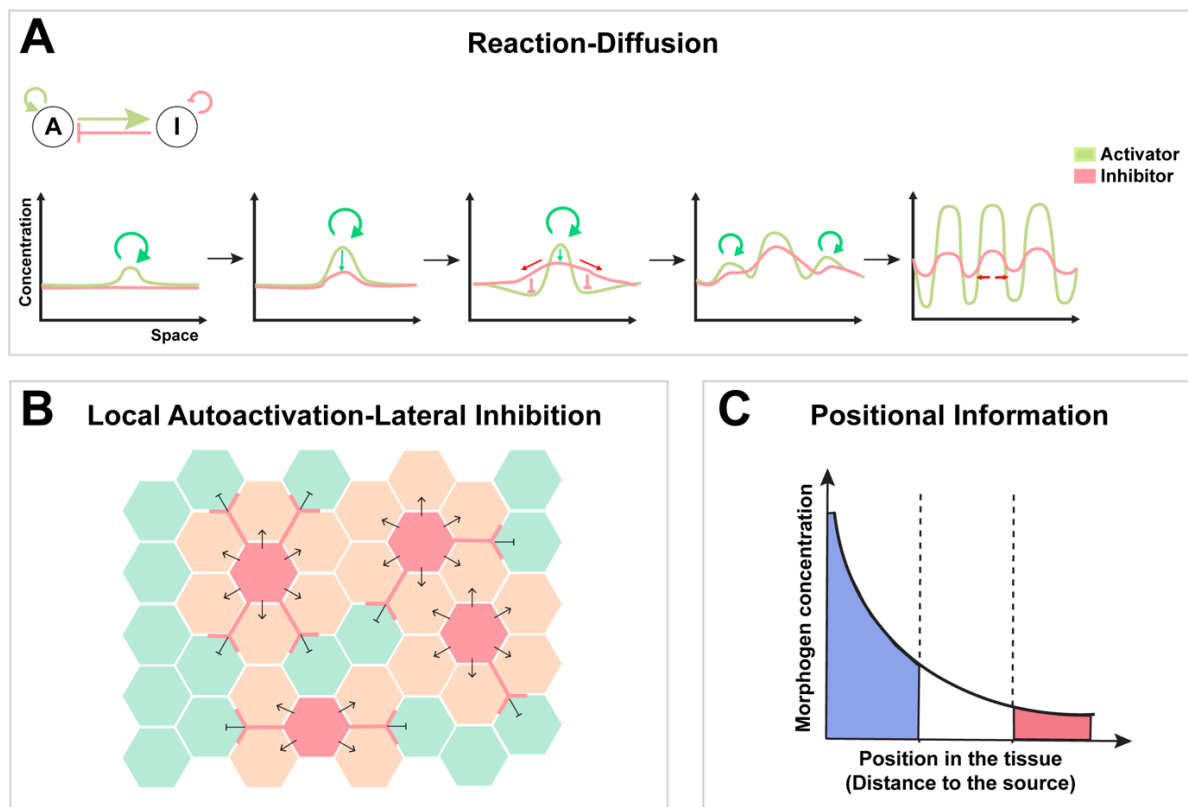


**Figure 4: Long-range mechanisms of self-organization.** **A:** Schematic of a classical reaction-diffusion model, describing how pattern in nature can arise from a homogeneous, uniform state by the interplay between two or more diffusible molecules. Initially, molecular fluctuations lead to a locally higher expression of an activator signal (green). This activator auto-induces in a feedback loop to maintain its own expression, but it also induces an inhibitor signal (red). The inhibitor diffuses faster than the activator signal, causing suppression of the activator between peaks, which leads to the formation of a periodic pattern in space with a consistent wavelength. **B:** Schematic of a local autoactivation-lateral inhibition model, in which activator or signal-sending cells (brick red) locally activate adjacent cells (peach) and inhibit further away located cells (turquoise), often mediated by cell protrusions. **C:** Schematic of the positional information or ‘French flag’ model, in which cells interpret their position based on a morphogen concentration gradient. Cells located close to the morphogen source receive higher concentrations of the morphogen than cells positioned further away in the tissue, leading to the establishment of multiple different cell fates.

## I.9 Short-range mechanisms

### I.9.1 Collective cell migration

Other mechanisms offer alternatives to morphogen gradients for generating spatial patterns of cells during development. One of those is collective cell migration, which refers to the coordinated movement of groups of cells in a tissue or organism (**Figure 5A**). In collective migration, cells maintain physical contact with neighboring cells as they move, often exhibiting cohesive behavior (Friedl & Gilmour, 2009). While the above-mentioned types of self-organization act on long-range, collective cell migration acts short-range due to the requirement for close cell-cell contacts. Cells may migrate as a cohesive group, such as epithelial cells, or maintain temporary cell-cell adhesion sites, as seen in mesenchymal cells (Weijer, 2009). During migration, cells can move as sheets, strands, clusters, or ducts instead of individually. They utilize similar actin- and myosin-mediated protrusions and are guided by external chemotactic and mechanical cues, like the mechanisms employed by single migratory cells (Friedl & Gilmour, 2009). Collective cell migration often involves cell sorting to a certain extent as cells re-arrange themselves during movement, however, cell sorting can also occur independently of migration. This process also requires cell-environment interactions, involving extracellular matrix proteins such as laminin, fibronectin, or collagen, which enable cells to adhere and migrate (Cerchiari et al., 2015). In the course of this process, cells form focal adhesions at the front and disassemble them in the back (Friedl & Gilmour, 2009; Mayor & Etienne-Manneville, 2016). Such cell-environment interactions are commonly used by neural crest cells after their delamination from the dorsal neural tube (Carmona-Fontaine et al., 2008). Additionally, collective cell migration requires cell-cell communication, either via the secretion of chemo-attractants that stimulate other cells in the group thus allowing them to move towards each other and maintain high cell density, or via contact inhibition of locomotion, in which a cell alters its direction of movement upon colliding with another cell (Fujimori et al., 2019). These cell-cell contacts are mostly mediated by specific transmembrane glycoproteins, so-called cadherins (Gumbiner, 2005). Another example in developmental biology that involves collective cell migration for self-organization other than neural crest cells is the lateral line formation in zebrafish embryos, which is essential for the development of the sensory system (Dalle Nogare et al., 2014).

### I.9.2 Sorting (based on attraction/affinity/adhesion)

An additional method of self-organization without the need of morphogen signaling involves the sorting of cells, where cells are initially specified and arranged in a mixed 'salt-and-pepper' fashion and subsequently physically re-arrange to form coherent, patterned tissues (**Figure 5B**) (Kay & Thompson, 2009; Krens & Heisenberg, 2011). Cell sorting is a short-range mechanism of self-organization, in which cells spatially re-arrange themselves based on specific properties such as adhesion, motility, or differential expression of cell surface molecules such as cadherins (Nicol et al., 1999; Xu et al., 1999; Maître et al., 2012). This process can take place within a tissue or between different tissues. Cell sorting does not involve or require positional information, however, it can provide a foundation for positional signaling and morphogen gradients to occur if the formed tissue acts as an organizer, as observed with SHH signaling in the zebrafish neural tube (Xiong et al., 2013). These results led the authors to challenge the traditional view of dorsoventral patterning in the neural tube, which is based on positional information and variations in transcription factor expression. They propose an alternative model where cell positions are initially unstable in the dynamic tissue, with morphogen signaling creating a spatial 'salt-and-pepper' pattern. Subsequently, different progenitors sort and segregate into defined domains.

Another example of cell sorting occurring during self-organization in development is observed in the specification of mouse trophectoderm ICM lineages, in which cells initially exhibit a random distribution of lineage markers, but subsequently organize themselves into distinct lineages through cell sorting (Chisholm & Houliston, 1987; Chazaud et al., 2006; Dietrich & Hiragi, 2007; Plusa et al., 2008; Chazaud & Yamanaka, 2016).

### I.9.3 Mechanochemical feedback

Mechanical forces play a crucial role in development by altering the size, shape, number, position, and gene expression of cells, which is essential for morphogenesis and tissue development (Nelson et al., 2005; Paluch & Heisenberg, 2009; Keller, 2012; Begemann et al., 2019). It is commonly accepted that changes in mechanical tissue feedback can drive pattern formation (Murray, 1981; Murray & Oster, 1984; Murray, 2003; Milinkovitch et al., 2013). Force transmission in tissue morphogenesis is determined by cell-environment (extracellular

matrix (ECM)) contacts such as integrins, as well as by cell-cell contacts such as cadherins (Heisenberg & Bellaïche, 2013), or by tissue bending respectively compression that subsequently lead to geometrical changes (Shyer et al., 2013; Hiscock & Megason, 2015). These forces are coordinated and integrated through long-range transmission and mechanosensing within tissues, leading to significant changes in tissue shape thus inducing the formation of spatial patterns and organization (**Figure 5C**) (Heisenberg & Bellaïche, 2013; Hannezo & Heisenberg, 2019). External mechanical forces can also regulate tissue patterning by influencing cell fate and differentiation. For example, during embryonic development, mechanical forces generated by cell movements and tissue deformation or curvature induction can provoke the secretion of morphogens, which establish concentration gradients that guide tissue patterning and morphogenesis (Tamemoto & Noguchi, 2020). Thus, the interaction between mechanical forces that are converted into biochemical signals, so-called mechanochemical feedback loops, contribute to self-organization.

An example of a signaling pathway that effectively uses these mechanochemical feedback loops is the Notch pathway (Sjöqvist & Andersson, 2019; Stassen et al., 2020; Dullweber & Erzberger, 2023). During cardiac trabeculation in zebrafish, Notch-dependent lateral inhibition regulates delamination and trabecular fate in neighboring cells, in which heterogeneity in local tension influences organ-scale patterning and cell-fate decisions (Priya et al., 2020). In this study, tissue-scale forces impact local cellular mechanics to shape complex structures and modulate cell-fate choices, by which multiscale regulatory interactions collectively ensure robust self-organized organ patterning.

Moreover, recent findings have demonstrated that the spatiotemporal and geometrical control of tissue growth *in vitro* induces symmetry-breaking and patterning of intestinal organoids through cell shape-mediated regulation of YAP and Notch signaling (Gjorevski et al., 2022). Even further, as already mentioned in “I.6.3 YAP signaling”, YAP1-mediated mechanotransduction induces notochord formation and ventral neural tube patterning in mouse embryos (Cheng et al., 2023), suggesting that self-organization is indeed supported by mechanochemical feedback loops.

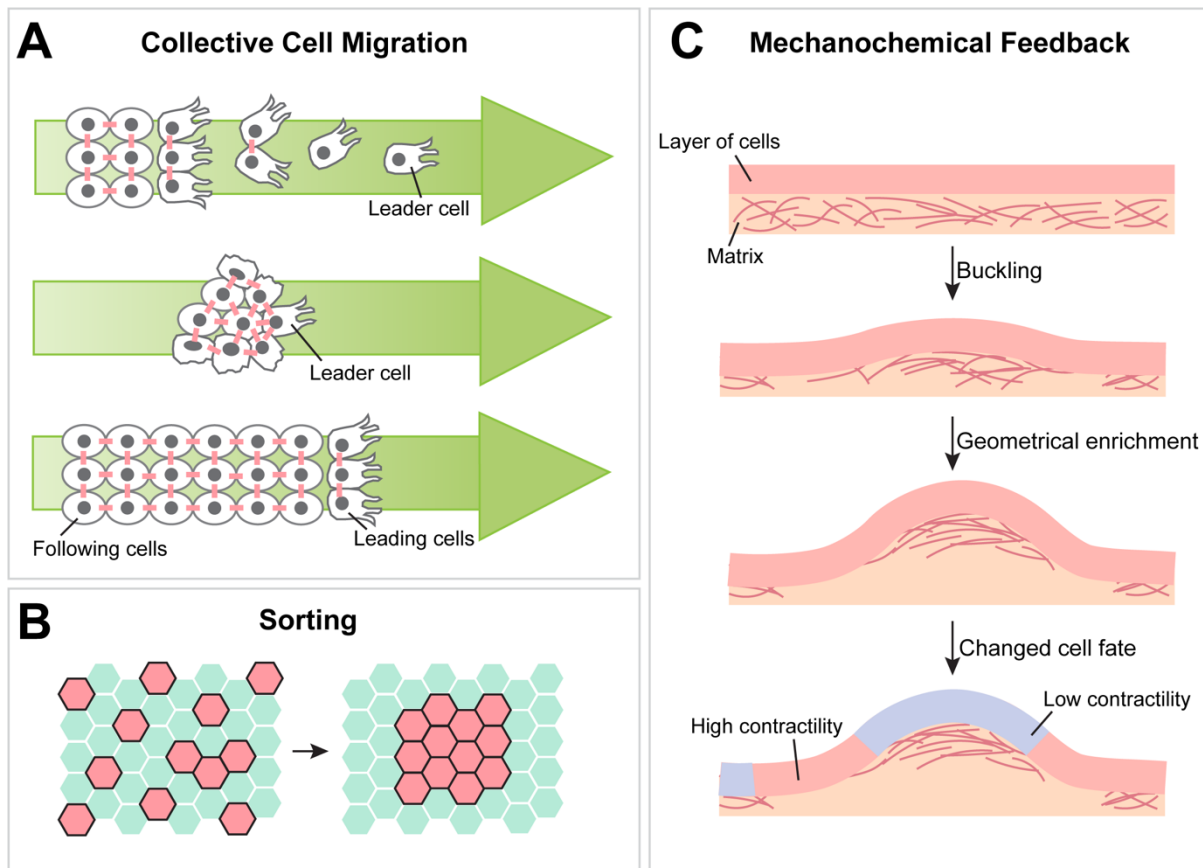


**Figure 5: Short-range mechanisms of self-organization.** **A:** Schematic of collective cell migration, where cells (white) travel as strands (top), clusters (middle), or sheets (bottom), following the leading cell(s). Rose-colored line represents direct cell-cell contacts. **B:** Schematic of physical cell sorting, where initially spatially scattered cells (rose) physically re-arrange to form coherent clusters. **C:** Schematic of mechanochemical feedback, where e.g. re-modelling of ECM molecules (brick red) leads to buckling and geometrical enrichment of cell layers, which results in force transmission and tissue morphogenesis.

## I.10 Organoids as a tool to study self-organization and pattern formation *in vitro*

Our extensive understanding of organizers, their formation and function during development, as well as the mechanisms of pattern induction and self-organization of complex tissues in developing vertebrates is largely due to precise and informative intra- and inter-species grafting studies, cell tracing, and tissue manipulations. However, in recent years, 3D stem cell models emerged as valuable tools that allow to study self-organization and formation of complex tissues in a precise and more controllable environment (Kretschmar & Clevers, 2016). These 3D structures such as organoids serve as simplified models that allow to link specific pathways to distinct events in early mammalian development, thereby providing a

versatile and physiologically relevant platform that bridges the gap between *in vitro* studies and *in vivo* conditions, which can enhance our understanding of tissue self-organization and pattern formation.

Organoids are formed from proliferating embryonic stem cells (ESCs), adult stem cells (ASCs) or induced pluripotent stem cells (iPSCs) from single cells (for example (A. Meinhardt et al., 2014)) or aggregates of cells (for example (Lancaster et al., 2013)). By culturing them under specific differentiation conditions comprised of medium and ECM typically supplied with (growth) factors, organoids can be directed to differentiate into various organ-specific cell types within defined developmental trajectories. Since these factors are present uniformly throughout the differentiation medium, they do not provide any spatial information or organization, thus organoids form through cellular re-arrangements and self-organization. Therefore, organoids represent exceptional tools for studying self-organization and pattern formation of tissues, also due to their ability to mimic *in vivo* conditions with remarkable reliability.

The 3D nature of organoids is crucial for observing spatial aspects of tissue organization, enabling cells to interact in a manner that is resembling processes occurring in the whole organism. Organoids recapitulate developmental processes such as differentiation, morphogenesis, and spatial patterning, making them ideal for studying these processes (Kretzschmar & Clevers, 2016). Since they replicate the architecture and functionality of actual organs, they offer a robust method for modeling tissue morphogenesis and organogenesis *ex vivo*. Furthermore, they facilitate the study of complex cell-cell and cell-matrix interactions, which are essential for maintaining tissue structure and function. They are also easily genetically and pharmacologically modifiable, offering a platform for high-throughput drug screening and allowing researchers to investigate the molecular mechanisms underlying tissue organization and identify potential therapeutic targets. Human-derived organoids, in particular, offer significant insights into human-specific developmental processes and disease mechanisms (Lancaster et al., 2013; Krenn et al., 2021; Eichmüller et al., 2022), providing a valuable tool for translational research. Additionally, advanced imaging techniques can be applied to organoids to monitor their development and organization in real time, offering dynamic insights into the temporal aspects of tissue formation and the

chronological events involved (Serra et al., 2019). Organoids further reduce the need of using animal models by addressing concerns related to animal testing. Finally, due to the relatively easy maintenance and expansion of stem cells, researchers have access to large amounts of test samples simultaneously.

Several organoid systems have demonstrated the ability to undergo symmetry breaking and self-organization, providing valuable insights into developmental processes and tissue architecture. Intestinal organoids, generated from ASCs or iPSCs, replicate the crypt-villus architecture of the intestine, where stem cells are located at the tip of the crypts and differentiated cells populate the villi, accurately reflecting the spatial organization of the intestinal epithelium (Sato et al., 2009; Serra et al., 2019). Gastruloids, aggregates of ESCs, model early embryonic post-implantation development stages, undergoing symmetry breaking to form anterior-posterior axes, somite formation, and early neural patterning, thereby recapitulating early embryogenesis and axis formation (Van Den Brink et al., 2014; Turner et al., 2017; Beccari et al., 2018). Cerebral organoids, derived from human iPSC or ESCs, develop complex brain-like structures with layered cortical regions, closely mimicking early brain development and regionalization (Lancaster et al., 2013; Lancaster & Knoblich, 2014a, 2014b).

### I.11. Neural tube organoids (NTOs)

NTOs, derived from mouse or human ESCs and PSCs, are able to mimic early neural tube development and recapitulate correct pattern formation (A. Meinhardt et al., 2014; Zheng et al., 2019; Veenvliet et al., 2020; Karzbrun et al., 2021; Park et al., 2022). These organoids reproduce key aspects of the developing embryonic neural tube, which is the precursor to the CNS. By using specific defined neurodifferentiation media, growth factors and signaling molecules, these organoids can be patterned to form distinct neural structures, allowing researchers to study neural development, investigate neural tube defects, and screen for potential therapeutic targets in a controllable environment. To date, several different protocols to form neural tube and spinal cord organoids, varying in starting cultures, differentiation medium, cell number and anterior-posterior identity, have been published (summarized in (Wu et al., 2021)). For example, researchers have successfully formed trunk-

like structures from neural tube tissue and somites (Veenvliet et al., 2020), patterned neuroepithelial cysts derived from human PSCs that showed low degrees of dorsoventral patterning (Zheng et al., 2019), or NTOs that fold upon neural induction due to geometric constraints (Karzbrun et al., 2021), effectively mimicking different key aspects of *in vivo* neurulation.

The first efficient method to generate NTOs in 3D that reproduced embryonic development and recapitulated full dorsoventral patterning, starting from a suspension of single mouse ESCs (**Figure 6A**), was published by Andrea Meinhardt and colleagues in the laboratory of Elly Tanaka (A. Meinhardt et al., 2014). In this work, mouse ESCs were dissociated and fully embedded as single cell suspension in Matrigel, a gelatinous protein mixture rich in ECM components that provides a scaffold which supports cell differentiation and growth, and put under defined neurodifferentiation conditions. NTOs grow clonally and form spherical or ellipsoid structures (**Figure 6B**), consisting of neuroepithelial cells with apical-basal cell polarity, as shown by apical expression of the markers PROMININ-1, ZO-1, and N-CADHERIN, organized around a single lumen. After seven days in culture, these NTOs are SOX1-positive (**Figure 6C**) and further uniformly express the neural stem cell markers NESTIN and MUSASHI, and additionally grow neurons basolaterally that are positive for the markers NeuN,  $\beta$ 3-TUBULIN, and MAP2a. By default, NTOs resemble dorsal neural plate progenitors as marked by PAX3, PAX7, and MSX1+2 expression, and are of anterior neural tube identity. Interestingly, a globally applied pulse of the signaling molecule RA posteriorized the NTOs to hindbrain-cervical spinal cord levels and leads to the formation of a localized, functional floorplate and consequently ventral-to-dorsal patterning with the formation of diverse types of neurons along the dorsoventral axis. Approximately 40% of the NTOs were patterned at the endpoint (A. Meinhardt et al., 2014), assessed by counter protein staining of the ventral floorplate markers FOXA2, SHH and OLIG2, and restricted expression of dorsal marker genes such as PAX3 on the opposite side of the NTO (**Figure 6D**).

This remarkable *in vitro* system forms the floorplate organizer through self-organization, however, the underlying mechanisms driving this process are poorly understood and remain to be determined.

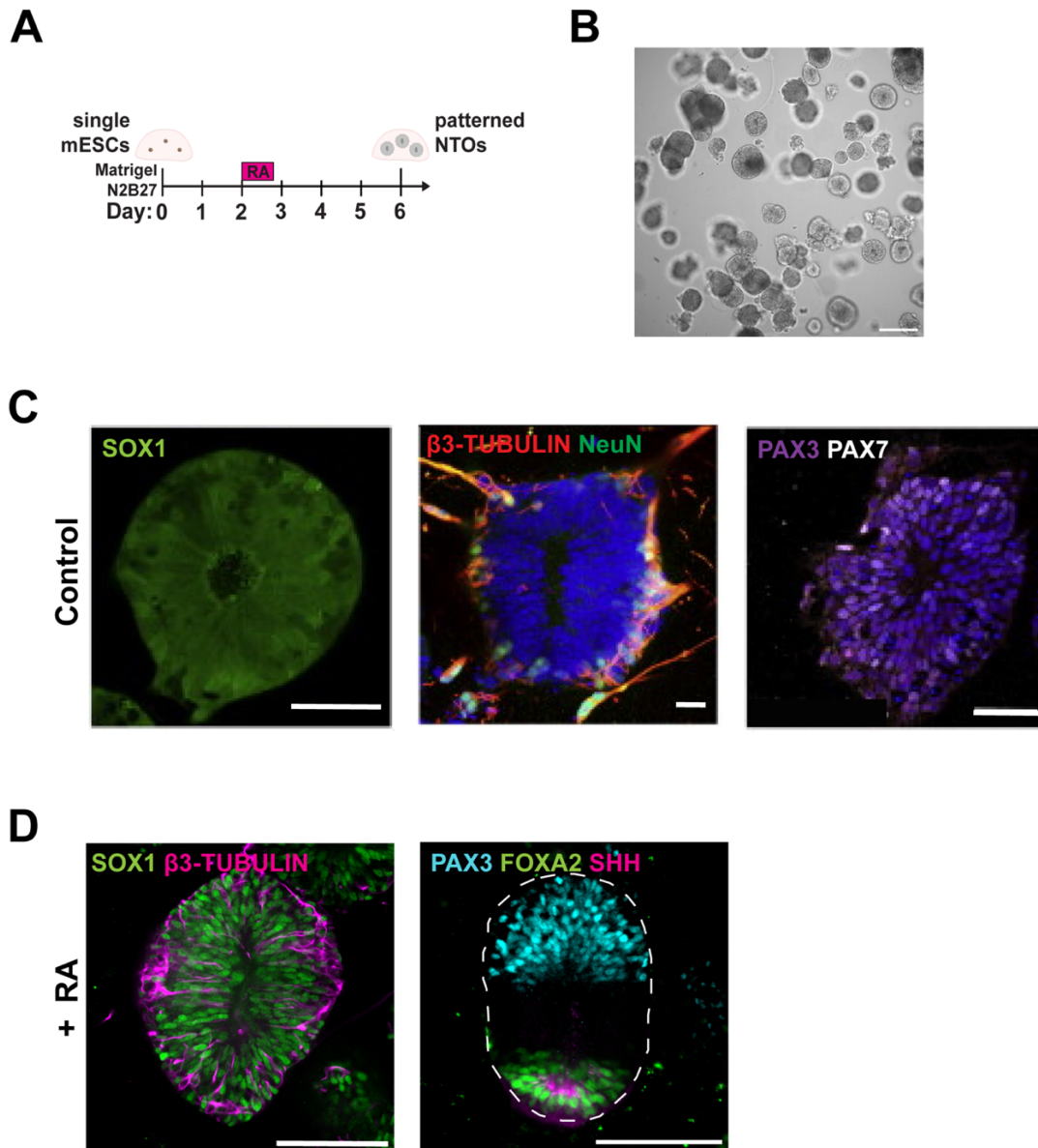


**Figure 6: Clonal NTOs from mouse ESCs.** **A:** Schematic of the NTO formation protocol. **B:** Day 6 NTOs treated with RA in brightfield. Scale bar, 200  $\mu$ m. **C:** Representative control NTOs on day 7 expressing the neural stem cell marker SOX1 (left), adult neuronal markers  $\beta$ 3-TUBULIN and NeuN (middle), and dorsal neural tube markers PAX3 and PAX7 (right). Scale bars, 50  $\mu$ m (left and right image) and 20  $\mu$ m (middle image). This figure panel was adapted from (A. Meinhardt et al., 2014). **D:** Representative RA-treated NTOs on day 6 expressing the neural stem cell marker SOX1 and the adult neuronal marker  $\beta$ 3-TUBULIN (left), as well as the dorsoventral patterning markers PAX3 (dorsal neural tube, dorsally restricted), FOXA2 and SHH (floorplate, ventrally restricted) (right). Scale bars, 100  $\mu$ m.

## II. Aims

The neural tube is a classic example of embryonic induction, where the notochord triggers the formation of an organizer, the floorplate, which then releases SHH to pattern nearby neural progenitors. In contrast, NTOs demonstrate spontaneous pattern formation *in vitro* without the usual embryonic inducers. In our laboratory, we have created a method that allows the formation of NTOs from single mouse ESCs, which can independently form a floorplate without a localized inducer through the process of self-organization.

The main goal of my PhD was to investigate how this organizing center forms in mouse NTOs. A global pulse of the signaling molecule RA can initiate the formation of a localized, fully functional floorplate by inducing the floorplate-specific transcription factor FOXA2. In turn, FOXA2<sup>+</sup> cells self-organize and induce SHH, which subsequently patterns the NTO in a similar way to what happens to the neural tube *in vivo*. Specifically, I asked:

1. How does FOXA2 expression transition from a spatially scattered to a clustered state?
2. Which mechanisms and/or pathways are involved in this process?
3. What is the *in vivo* relevance of NTO floorplate-self organization?

To address these questions, I first optimized the NTO protocol to, among other improvements, increase ventral patterning efficiency. This optimization enabled me to then study the mechanism by which a global pulse of RA results in the formation of a single ventral signaling center per organoid, the floorplate, using a combination of live imaging, drug screening, pathway localization analysis and genetic manipulations. The findings of this work are presented as two research papers published in international journals.



### III. Results

The publication and the manuscript presented in III.1 “Neural tube organoid generation: a robust and reproducible protocol from single mouse embryonic stem cells” (BioRxiv, (Krammer & Tanaka, 2024)) and III.2 “Mouse neural tube organoids self-organize floorplate through BMP-mediated cluster competition” (Developmental Cell, (Krammer et al., 2024)) have been either submitted for publication or are already published. The manuscript in III.1 has not yet been peer-reviewed and may therefore change. Confirmation that both papers have been submitted to international journals can be found at the beginning of sections III.1 and III.2.



### III.1 “Neural tube organoid generation: a robust and reproducible protocol from single mouse embryonic stem cells”

3D stem cell models, such as organoids, are valuable tools to study key developmental questions in a precise context. Neural organoids, specifically, have significantly enhanced our understanding of neural tube, spinal cord, and brain development, as well as the causes of associated diseases. To minimize heterogeneity in experimental outcomes and to increase reproducibility, detailed protocols are essential. Therefore, one of our first efforts was to optimize the protocol, which has been previously established by the Tanaka lab, to allow for reliable and reproducible neural tube organoid (NTO) generation. Using this protocol, NTOs show increased cell viability, faster epithelialization and higher ventral patterning efficiency.

|                       |                                                                                                                                                                                                     |
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| <b>Status:</b>        | Submitted to the preprint server for Biology<br>bioRxiv.org<br>Publication date: 22.06.2024                                                                                                         |
| <b>Authors:</b>       | <u><b>Teresa Krammer</b></u> , Elly M Tanaka                                                                                                                                                        |
| <b>Contributions:</b> | <u><b>T.K.</b></u> designed and performed experiments, created methodologies, and analyzed the data. <u><b>T.K.</b></u> and E.M.T. wrote the manuscript. E.M.T. conceived and supervised the study. |
| <b>DOI:</b>           | 10.1101/2024.06.20.599764                                                                                                                                                                           |

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# Neural tube organoid generation: a robust and reproducible protocol from single mouse embryonic stem cells

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## SUMMARY:

Three-dimensional neural tube organoids (NTOs) derived from mouse embryonic stem cells are valuable tools to study the central nervous system during early development. Here, we present a step-by-step demonstration of an optimized protocol for cultivating NTOs *in vitro*, providing stable culture conditions and troubleshooting strategies for reliable and reproducible NTO generation.

## ABSTRACT:

The development of mammals is a highly complex process, characterized by the necessity for precise concentration- and time- dependent signaling for correct pattern formation and morphogenesis. Despite considerable technological advancements and knowledge gathered, numerous aspects of mammalian development remain elusive. When examining the entire organism, it becomes challenging to disentangle the effects of individual pathways or the mechanism by which external stimuli guide the interference of surrounding tissues and factors. In addressing this complexity, three-dimensional (3D) *in vitro* models such as organoids have emerged as valuable tools.

Organoids, derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), exhibit tissue-like features that closely resemble their *in vivo* counterparts in terms of expression patterns and functionality. Importantly, they offer accessibility for manipulation and extensive biological studies within a controlled experimental setting. Despite originating from pluripotent cultures, organoid systems often exhibit heterogeneity and substantial variability, limiting their utility when studying complex and intricate biological questions. Therefore, there is a pressing need for detailed protocols aimed at harmonizing procedures that result in high-quality reproducible data, reduction of materials used and which importantly permit the investigation of convoluted phenomena. In this context, we present an optimized protocol for the cultivation of neural tube organoids (NTOs) *in vitro*. By producing stable culture conditions and offering comprehensive troubleshooting strategies, this protocol enables the reliable and reproducible generation of NTOs which serve as an adequate model to study relevant scientific questions.

## INTRODUCTION:

Mammalian development, starting from a single totipotent stem cell, is a complex process that involves the induction of pattern formation and morphogenesis. This development requires tightly regulated cellular programs to manage dynamic interactions between cells and their environments. Stem cells have the intrinsic ability to sense, integrate, and respond to systemic and local signals including morphogen gradients <sup>1</sup>, mechanical boundaries <sup>2</sup>, cellular proliferation, and environmental remodeling <sup>3</sup>. External spatiotemporal cues also drive multicellular responses, initially, in homogenous tissues, to ensure the precise formation of complex structures. Stem cells, distinguished by their self-renewal <sup>4, 5</sup> and differentiation capabilities, play a crucial role in embryonic development and adult tissue repair, with main types including embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs).

ESCs are extracted from the inner cell mass (ICM) of a blastocyst and are typically cultured in a two-dimensional (2D) environment on feeder cells or extracellular matrix using specific culture media to maintain their pluripotency <sup>6</sup>. In recent years, the need for more realistic cell culture conditions and technologies increased drastically, thus

three-dimensional (3D) cell and tissue models were established to study fundamental biomedical processes. Especially in developmental biology, these 3D models such as organoids offer a robust method for modeling tissue morphogenesis and organogenesis *ex vivo* <sup>7</sup>. Organoids form through self-organization of proliferating stem cells that spontaneously develop into complex 3D structures through symmetry breaking and pattern formation. This process is similar, though not identical to what is observed *in vivo*, and mostly occurs without external guidance but driven by internal dynamics and interactions <sup>8</sup>. Since organoids closely mimic the structure and function of actual organs, they can provide a more accurate representation of mammalian biology compared to traditional 2D cell cultures. In addition, one of the primary benefits is the ethical advantage, as organoids reduce the need for animal models, addressing concerns related to animal testing. Furthermore, organoids offer a platform for high-throughput drug screening, improving the prediction of drug efficacy and toxicity. However, there are also several challenges associated with using organoids. While they are more realistic than 2D cultures, they do not fully replicate the complexity and interactions of entire organs within a living organism. Additionally, there can be significant variability observed between organoids, even when derived from the same type of cells, leading to inconsistencies in experimental results. Thus, culturing organoids in a robust and reproducible manner is essential, and requires advanced technical skills, specialized equipment and detailed protocols.

The nervous system is a complex network responsible for coordinating actions and processing sensory information by sending and receiving signals throughout the body, thereby controlling and coordinating all required functions, including movement, sensation, thinking, and autonomic functions such as heartbeat and digestion. The precursor for the central nervous system, consisting of the brain and spinal cord, is the neural tube. It is crucial to understand how neural structures form and function within early neural tube development, to obtain insights into various neurological disorders and developmental abnormalities. Neurodevelopmental disorders are challenging to study *in vivo*, especially when they originate from human subjects due to ethical concerns. Neural organoids such as brain organoids <sup>9</sup> have significantly enhanced our understanding of nervous system development and disease by providing versatile, accessible models that closely mimic human brain tissue. These three-dimensional structures, derived from pluripotent stem cells (PSCs), replicate various aspects of

brain development, including the formation of distinct brain regions, cellular diversity, and complex neural networks<sup>10, 11</sup>. By allowing researchers to observe developmental stages in real-time and study key regulatory mechanisms, these organoids offer deep insights into normal brain development. Brain organoids also enable the modeling of neurodevelopmental<sup>12</sup> and neurodegenerative diseases<sup>13</sup>, such as microcephaly<sup>10, 14</sup>, Zika virus infection<sup>15</sup>, autism spectrum disorders<sup>16</sup> and others, elucidating disease mechanisms and identifying potential therapeutic targets. Additionally, brain organoids serve as valuable platforms for drug testing and development, facilitating the assessment of drug efficacy and toxicity in a human brain-like environment and accelerating personalized medicine approaches. Moreover, they provide a controlled setting to study gene-environment interactions, enhancing our understanding of complex diseases resulting from the interplay of genetic and environmental factors. However, since neural organoids, like brain organoids, are typically formed from aggregates of cells, they often exhibit significant cellular heterogeneity and variability in experimental outcomes, which in turn influences the interpretability of the generated data, posing a considerable challenge for researchers. For this reason, protocol optimization and precise quality control criteria are critical for the attenuation of valuable data that adequately represent the *in vivo* condition.

Meinhardt and colleagues<sup>17</sup> made a crucial impact by first reporting a clonal neural organoid protocol mimicking early neural tube development, formed from single embryonic stem cells (ESCs) that drastically minimizes inter-organoid heterogeneity. This innovation allows researchers to study developmental processes and mechanisms, as well as neural patterning, in a precise and defined environment with exceptional resolution. Until now, many laboratories have developed neural organoids, mostly of mouse or human origin, that mimic different parts of the neural tube and its development, as summarized in<sup>18</sup>. However, to our knowledge, the protocol established by Meinhardt et al.<sup>17</sup> is the only method where NTOs reliably form from a single cell, self-organize and efficiently pattern into dorsoventral fates following a single, global pulse of retinoic acid (RA) without the addition of other ventralizing factors such as Sonic Hedgehog Agonist (SAG)<sup>19</sup>, or the usage of microfluidic chambers that provide spatial information due to gradient formation. The protocol provided here was adapted from Meinhardt et al.<sup>17</sup> as provided in Krammer et al.<sup>19</sup>. In this protocol, single mouse ESCs are seeded in Matrigel and exposed to defined

neurodifferentiation conditions. Within two days, these cells clonally expand and epithelialize, forming a 3D round-to-ellipsoid structure with a single lumen and apically-basally stratified cells surrounding it. On day 2, a globally applied 18-hour pulse of the signaling molecule RA enhances neural differentiation, posteriorizes the NTOs from midbrain to hindbrain/cervical spinal cord levels, and induces the formation of a single, functional floorplate by day 6, which patterns the NTO from ventral to dorsal.

The protocol has the following advantages:

- Robust epithelialization within first 3 days,
- Relatively small spherical tissues (~200  $\mu\text{m}$ ) with a single lumen,
- Clonal growth from single cells,
- Serum-free, chemically defined culture medium,
- Gradual neural differentiation from pluripotent cultures within 6 days,
- 'By-default' anterior midbrain identity by day 6,
- Dorsoventrally patterned hindbrain/ cervical spinal cord-like structures within 6 days <sup>19</sup>.

Several aspects have been adapted and differ from the original protocol <sup>17, 20</sup> in the following ways:

- Different ESC culture condition,
- Less input cells,
- Improved differentiation medium,
- Changed differentiation protocol (no NOG d0-2) (already used in <sup>19</sup>),
- Seeding (density) adapted to multi-well plate (96) usage for high throughput analysis leading to 40% increase in NTO formation and patterning efficiency (from initially ~40% to now ~80%).

Here, we provide a detailed description of an optimized protocol for a robust and reproducible generation of NTOs in Matrigel for:

- NTO handling in all stages from seeding ESCs for differentiation assay, to maintenance, fixation and analysis.
- Quality control by visual assessment of morphology of NTOs on daily basis.
- Critical characterization of correct NTO identity via antibody staining.

In addition, we offer comprehensive troubleshooting approaches, allowing a critical assessment in every step of the protocol, which is necessary to ensure the reproducibility and reliability of experimental protocols. These detailed instructions help to identify and resolve potential issues, minimize errors, and optimize outcomes. This is crucial for maintaining the integrity of scientific experiments and enabling other scientists to successfully replicate the work and draw comprehensive conclusions from the data generated, thereby advancing the field.

# PROTOCOL:

## General statements:

- Cells and NTOs are checked daily for quality assessment before any further procedure (medium replacement, treatment, fixation, etc.).
- The morphology of the NTOs is crucial and can provide significant insights into the viability of the culture and the efficacy of the experiment.
- All reagents (Medium, PBS, Accutase) are pre-warmed at a minimum of 21 °C and maximum 37 °C before use.
- When preparing medium (N2B27, N2B27diff) glutamine is kept for a maximum of 5-10 minutes at 37 °C, better at room temperature.
- All procedures requiring direct exposure of cells to air must be performed within a biosafety cabinet to ensure sterility and prevent contamination of the culture.
- Cells and NTOs are kept out of the incubator for as limited time as possible and all required steps need to be taken in a careful and timely manner.
- Only good quality ESCs (Figure 2A-C) form good quality NTOs (Figure 4B).
- If more than 10% of mouse ESCs are differentiated (Figure 2D), the culture is discarded and freshly thawed culture is prepared which is passaged at least twice before seeding ESCs for a differentiation assay (experienced researchers can judge by visual assessment (see also Mulas et al. <sup>21</sup>) or via antibody staining for marker expression (e.g. OCT4, see also Kalkan et al. <sup>22</sup>)).
- Cells and NTOs are kept without medium as briefly as possible to avoid drying out the culture and/or Matrigel.
- The optimal seeding density is recommended (Table 2 and Figure 3). However, for certain applications, such as early analysis, a seeding density up to 10 times higher may be advantageous.
- If NTOs look suboptimal (Figure 4C-E) or do not show correct spatiotemporal marker expression (Figures 5 & 6), the material does not meet quality standards and is discarded.

# **1. Maintenance passaging and seeding mouse ESCs for NTO differentiation**

## **1.1. Passaging**

NOTE:

- ESCs are maintained on cell-culture dishes or wells of a 6-well plate pre-coated with 0.15% gelatin for 30 minutes at 37 °C.
- ESCs are passaged every 2-3 days, with the medium being replaced daily from day 2 onwards.

**1.1.1.** Check cells under phase-contrast microscope (see representative images, Figure 2).

NOTE:

- Ensure that colonies have an appropriate size and proper morphology (Figure 2A), while also confirming that they adhere to the bottom of the dish.

**1.1.2.** Open the lid of the culture dish/ plate, slightly tilt it and carefully aspirate medium.

**1.1.3.** Wash colonies with 500 µl pre-warmed PBS (added to cells in a single well of a 6-well plate), add and then aspirate again immediately.

NOTE:

- This step is crucial because: A) ESC medium (2iLIF) carry over must be avoided prior to the differentiation assay (or NTO formation will be hindered), B) FBS or BSA in culture medium can stop dissociation. This applies to ESCs cultured in a different ESC medium, containing FBS or BSA.

**1.1.4.** Add 500 µl pre-warmed Accutase and transfer cells back into the incubator.

**1.1.5.** Incubate cells at 37 °C for 3 minutes using a timer.

**1.1.6.** After the incubation time, take out the plate and check cells under the microscope.

NOTE:

- ESCs should have detached from the culture plate, if not tap the dish/plate firmly but carefully (Figure 2B).

**1.1.7.** Add 1 ml N2B27 medium by pipetting it directly onto to cells.

**NOTE:**

- ESCs should be washed off the plate, leaving 'empty' areas in the culture dish.
- 1.1.8.** Take a P1000 pipette, tilt the plate slightly such that all the colonies sticking to the culture dish can be washed off.
  - 1.1.9.** To dissociate clumps of cells, tilt the plate slightly, place the pipette tip (P1000) in the middle of the dish. Press the tip against the bottom of the dish but slightly off the perpendicular axis and flush out the cells. Repeat for five to ten times to break the cell clumps.
  - 1.1.10.** Check that the cells are dissociated properly into single cells under the microscope (Figure 2C), repeat step 1.1.9. if there are still clumps.
  - 1.1.11.** After complete dissociation, transfer the cell suspension into a clean sterile 15 ml Falcon tube, wash the empty well where cells were cultured in with 1 ml N2B27 medium and transfer to the same Falcon tube with the cell suspension.
  - 1.1.12.** Spin down cells for 5 minutes at 1500 rpm in an ultra centrifuge.
  - 1.1.13.** Carefully aspirate the supernatant.
  - 1.1.14.** Resuspend the cells in an appropriate amount of N2B27 medium (e.g. 1 ml) and count cells (see Table 2 for recommended cell numbers for differentiation assay into NTOs).

## **1.2. Differentiation of ESCs into neural tube organoids (NTOs)**

### **1.2.1. Aliquoting Matrigel**

- 1.2.1.1.** Thaw bottle of Matrigel overnight on ice at 4 °C.

**NOTE:**

- Best to do this in a box with ice at 4 °C or in the fridge
  - Do not leave Matrigel for longer than necessary at 4 °C, aliquot and freeze as soon as possible (otherwise this can affect the functionality of the Matrigel)
- 1.2.1.2.** In the biosafety cabinet: once the Matrigel is thawed, place 1.5-2 ml Eppendorf tubes on ice to pre-cool them, while also keeping the Matrigel bottle on fresh ice.

- 1.2.1.3.** Mix the thawed Matrigel carefully to avoid bubble formation.
- 1.2.1.4.** Aliquot Matrigel at the desired volume (0.5-1 ml is recommended) and freeze at -20 °C until further usage.

**NOTE:**

- Do not replace the tip between the aliquots, these are 'warm' (room temperature) and will cause the Matrigel to jellify within, which leads to a significant loss of Matrigel.
- Instead, cool the pipette tips to 4 °C by putting them in the fridge before use.

**1.2.2. Seeding single cells in Matrigel**

- 1.2.2.1.** Quantify the cell number needed (100 cells/ µl Matrigel, see Table 2).
- 1.2.2.2.** Transfer appropriate cell number (cell suspension) to a clean 15ml Falcon tube and top it up with 1ml of N2B27.
- 1.2.2.3.** Spin down cells for 5 minutes at 1500 rpm in the centrifuge.
- 1.2.2.4.** Aspirate the supernatant and place Falcon tube on ice.
- 1.2.2.5.** Carefully resuspend the cell pellet on ice in an appropriate amount of thawed and liquid Matrigel while avoiding the formation of bubbles.
- 1.2.2.6.** Spread the appropriate amount of Matrigel-cell suspension, in a thin layer, onto a glass bottom or plastic bottom dish which is optimal for imaging (see Table 2, Figure 3A-C).

**NOTE:**

- Make sure not to touch the walls of multi-well plates.
  - If using multi-well plates: best to first draw a circle of Matrigel using a P20 (96-well plate) or P200 pipette tip before 'filling it' with the Matrigel-cell suspension (Figure 3B).
- 1.2.2.7.** Put the multi-well plate/ dish in the incubator at 37°C to let the Matrigel jellify (rule of thumb: 1 minute per µl Matrigel for smaller amounts and a maximum of 15 minutes for 40 µl or more) (use a timer).
  - 1.2.2.8.** After the incubation time, take out the plate and add appropriate volume of medium (see Table 2).

### 1.2.2.9. Check the seeding density and cell distribution within the Matrigel under the microscope (Figure 3C-D).

#### NOTE:

- Keep Matrigel aliquot on ice or in the fridge, otherwise it will jellify at room temperature.
- Carefully mix the Matrigel-cell suspension by resuspending with a P300 or P1000 pipette tip before seeding for differentiation assay, otherwise cell number will be highly variable between wells/ dishes (not recommended to do before seeding every single well, because this leads to loss of Matrigel as Matrigel can jellify and stick to the pipette tip).
- The presence of bubbles into the Matrigel can cause its detachment off the well/ dish after jellification.
- ESCs in Matrigel should not dry out during seeding for differentiation assay into NTOs or replacing medium. After jellification, medium should be applied as soon as possible. Jellification typically is complete in 1 minute at 37 °C per 1 µl Matrigel and maximum 15 minutes for 40 – 100 µl Matrigel when spread as a thin layer.
- Because of high surface tension, some multi-well plates can cause the Matrigel droplet to move from the center of the well to the walls if the well walls are touched, which causes an inadequate distribution of cells/ NTOs.
- Matrigel should always be thawed on ice or 4 °C (never at room temperature or in hands).

## 2. RA treatment of NTOs to enhance neural differentiation and to induce posteriorization and ventralization

### 2.1. Aliquoting retinoic acid (RA)

- 2.1.1. Bring all-trans RA powder to room temperature (stored at -20 °C).
- 2.1.2. Reconstitute powder in an appropriate volume of DMSO to reach a final concentration of 100 mM.
- 2.1.3. Aliquot 1 µl of 100 mM RA into one 1.5 ml screw-top vial each.
- 2.1.4. Add Argon gas until tube is 'full'.

**NOTE:**

- Since argon gas is heavier than oxygen, it will sink to the bottom of the tube to cover and preserve RA from air. This can be felt on hands when it leaks out when tube is full.

**2.1.5.** Tightly close the vial.

**2.1.6.** Store aliquots in a black box at -80 °C until further usage (alternatively: aliquot in Eppendorf tubes and store in a black box in liquid N<sub>2</sub>).

**NOTE:**

- RA is highly sensitive to light and air.
- Keep thawed RA aliquots always in the dark.
- Reconstituted and diluted RA can be stored in the fridge (covered with foil) for up to 2 days (after that, discard and use a fresh aliquot).

**2.2. RA treatment of NTOs for 18h**

**2.2.1.** Take out 1 µl aliquot with a concentration of 100 mM from -80 °C and keep dark until thawed on room temperature (palm of hand or in tube rack in biosafety cabinet without lights on).

**NOTE:**

- If RA aliquots are stored in liquid N<sub>2</sub>, immediately open the lid of the Eppendorf tube to avoid explosion of the tube, then close again and proceed to step 2.2.2.

**2.2.2.** Once thawed, add 400 µl of pre-warmed N2B27diff medium for a concentration of 250 µM using a P1000 pipette. Mix thoroughly to achieve homogeneity.

**NOTE:**

- Alternatively, RA can be reconstituted and diluted in pure DMSO instead of N2B27diff medium, although this increases the concentration of DMSO in the culture.

**2.2.3.** Use the dilution from 2.2.2. in a ratio of 1:1000 in N2B27diff to get an appropriate volume with a final concentration of 250 nM RA.

- 2.2.4.** Take out plates/ dishes growing NTOs from the incubator and place into a sterile biosafety cabinet.
- 2.2.5.** Remove the lid of the plate/ dish and slightly tilt the plate to the side.
- 2.2.6.** Aspirate the medium carefully either using a P200 pipette, or an aspirator without disturbing the Matrigel.
- 2.2.7.** Add an appropriate volume of N2B27diff supplemented with 250 nM RA solution (see Table 2), cover with the lid and transfer the NTOs back into the incubator.

**NOTE:**

- Sometimes, especially if the RA was not mixed completely, precipitates can form that are harder to dissolve in normal N2B27diff (then, use DMSO). In this case it is advisable to discard the aliquot and make fresh RA aliquots because these precipitates have an unknown and uncontrolled concentration of RA.
- Make sure ESCs in Matrigel do not dry out while replacing medium or treating with RA (handle a maximum of 8 wells of 96-well plate if using a multichannel pipette, 1 MatTek dish/ well of any other multi-well plate)

### **3. RA removal and routine of daily medium replacement of NTOs**

- 3.1.** Transfer the culture plate from the incubator and move to the sterile biosafety cabinet.
- 3.2.** Remove the lid and slightly tilt the plate to the side.
- 3.3.** Carefully aspirate N2B27diff containing RA either using a P200 pipette, or aspirator without touching the Matrigel.
- 3.4.** Add pre-warmed PBS (add enough volume to cover the Matrigel droplet, or use volume as referred to in Table 2), then tilt the plate again and carefully remove it to wash out the leftover RA.
- 3.5.** Add appropriate volume of N2B27diff by pipetting the medium at the wall of the well/ dish to not destroy the Matrigel droplet.
- 3.6.** Replace medium daily following the steps 3.1. – 3.3., then adding fresh pre-warmed N2B27diff by pipetting the medium onto the wall of the well/ dish before returning the NTOs back in the incubator.

**NOTE:**

- Direct and vigorous pipetting directly or addition of cold medium/ PBS onto the Matrigel droplet can cause its breakage and detachment from the plate/dish.
- When perturbing pathways by using small molecules or recombinant proteins, it is important to replace the medium daily. If drugs are to be applied for longer than one day, drug solutions need to be prepared freshly daily or at least every two days to ensure protein/ drug stability and the continued presence of correct concentrations.

#### **4. Fixation of NTOs**

**NOTE:**

- NTOs are fixed as described by Krammer et al.<sup>19</sup>. Depending on the antibody to be subsequently used, PFA from different sources (store bought, in-house, etc.) can be required.

- 4.1.** Thaw PFA aliquot at 4 °C overnight.
- 4.2.** Bring PFA to room temperature before use.
- 4.3.** If using a higher stock concentration of PFA, dilute PFA to 4% in N2B27diff medium or PBS in a chemical hood.
- 4.4.** Take NTOs out of the incubator and transfer them to a chemical hood.
- 4.5.** Open the lid and add 4% PFA in a ratio of 1:1 directly to the NTOs to result in a final concentration of 2% (e.g. 100 µl 4% PFA added to the 100 µl N2B27diff in the 96-well plate).
- 4.6.** Put the lid back on and incubate NTOs for 30 minutes at room temperature (set a timer).

**NOTE:**

- PFA is toxic and should not be inhaled. Work carefully and with caution under the chemical hood.
  - Longer fixation can impair performance of certain antibody treatment.
  - Some antibodies require usage of a specific PFA or shorter/ optimized fixation time.
- 4.7.** After 30 minutes, remove the lid and slightly tilt the plate to the side, then follow steps 3.3. – 3.4.

- 4.8. Carefully add pre-warmed PBS (add enough volume to cover the Matrigel droplet or use volume as referred to in Table 2), and incubate for 5 minutes at room temperature, repeat this step 3 times to remove all left over PFA.
- 4.9. After the final wash, add appropriate amount of PBS (see Table 2), seal the plate using parafilm and store at 4 °C until further usage.

## 5. Antibody staining of NTOs

### NOTE:

- Antibody staining is a valuable in assessing the correct identity of the NTOs as well as the success of the assay/ experiment.
- Antibody staining can be performed as outlined in Meinhardt et al.<sup>17</sup> and Krammer et al.<sup>19</sup>
- Block/ permeabilization solution (1x PBS with 1% BSA and 0.5% Triton-X) is kept at 4 °C and adapted to room temperature before use.
- For a full 3D reconstitution and analysis after imaging, antibodies (1<sup>st</sup> and 2<sup>nd</sup>) should be kept at least 1, but preferably 2 – 3 nights at 4 °C to ensure good antibody penetration.
- Alexa Fluor anti-561 2<sup>nd</sup> antibody might give stronger background signal in Matrigel in the first three days of the differentiation assay.

## 6. Clearing of NTOs

### NOTE:

- NTOs can be cleared using the protocol from Krammer et al.<sup>19</sup>. The addition of propylgallate to the clearing solution minimizes photobleaching of the NTOs.
- Clear NTOs only after completing the antibody staining process (1<sup>st</sup> and 2<sup>nd</sup> antibodies are incubated and washed out properly).
- Perform clearing approximately 1-2 hours before imaging to prevent oxidization of the clearing solution (liquid turns from light yellow/cream into dark bright yellow or even brown) or crystallization in the well.
- Crystallized clearing solution can be dissolved by the addition of PBS.

- Correct pH of the clearing solution is crucial, otherwise Matrigel can dissolve or clearing solution can oxidize at a faster rate.
- Avoid shaking or mixing the clearing solution harshly because this results in the formation of air bubbles.

## 7. Analysis of NTOs

To analyze the NTOs after full penetration of antibody staining and whole tissue clearing, we recommend using a confocal microscope with 10-40x objectives. For 3D analysis, the software CellProfiler <sup>23, 24</sup>, Imaris or similar can be used. Verifying the correct identity of NTOs and the spatiotemporal expression of specific dorsoventral patterning markers is essential to determine whether the differentiation assay has been successful before addressing fundamental research questions. In Figures 5 and 6, we show characterization of three essential aspects to determine the success of the culture:

- 1) Epithelialization and lumen formation (ZO-1)
- 2) Neural differentiation (OCT4, SOX1,  $\beta$ 3-tubulin) (in-depth characterization of neural differentiation trajectory in <sup>17</sup>)
- 3) Dorsoventral patterning (FOXA2, SHH, PAX3) (in-depth characterization of ventral patterning in <sup>19</sup>)

## REPRESENTATIVE RESULTS:

R1 mouse ESCs were used for all representative examples in figures presented in this manuscript. Other mentioned mouse ESCs can exhibit mild differences in epithelialization timing, neural differentiation or patterning efficiency when seeded for NTOs, however, results should not significantly differ from the data shown here.

When good quality ESCs (Figure 2) are seeded at the appropriate density (Figure 3) in Matrigel, these single cells clonally expand and epithelialize within two days (Figure 4). NTOs exhibit a smooth, round-to-ellipsoid shape with a single lumen. NTOs that have failed to epithelialize lack lumen formation, with individual cells remaining visible. Small round debris observed around the NTOs are apoptotic bodies, indicative of a stressed culture.

On day 2, NTOs are negative for any neural (SOX1) and neuronal ( $\beta$ 3 Tubulin) marker, as well as dorsoventral patterning markers (FOXA2, PAX3, SHH), but are expressing ZO-1 and OCT4 (Figure 5). On day 2, a 250 nM RA pulse is applied for 18 hours to ventralize the NTOs to hindbrain/ cervical spinal cord levels and to boost neural differentiation<sup>17, 19</sup>. From day 2 onwards, NTOs gradually downregulate the pluripotency marker OCT4 and from day 3 onwards, they start expressing SOX1 and subsequently  $\beta$ 3 Tubulin by day 6 (Figure 6). RA treatment accelerates neural differentiation compared to NTOs in N2B27diff only. Full dorsoventral patterning markers (FOXA2, SHH, PAX3) are only expressed after RA treatment, while without RA, NTOs will become positive for PAX3 and SOX1 by day 6 (Figure 6). RA-treated NTOs exhibit a smaller and more compact lumen compared to those in N2B27diff, as demonstrated by the tight-junction marker ZO-1 (Figures 4 and 6).

**Figure 1**

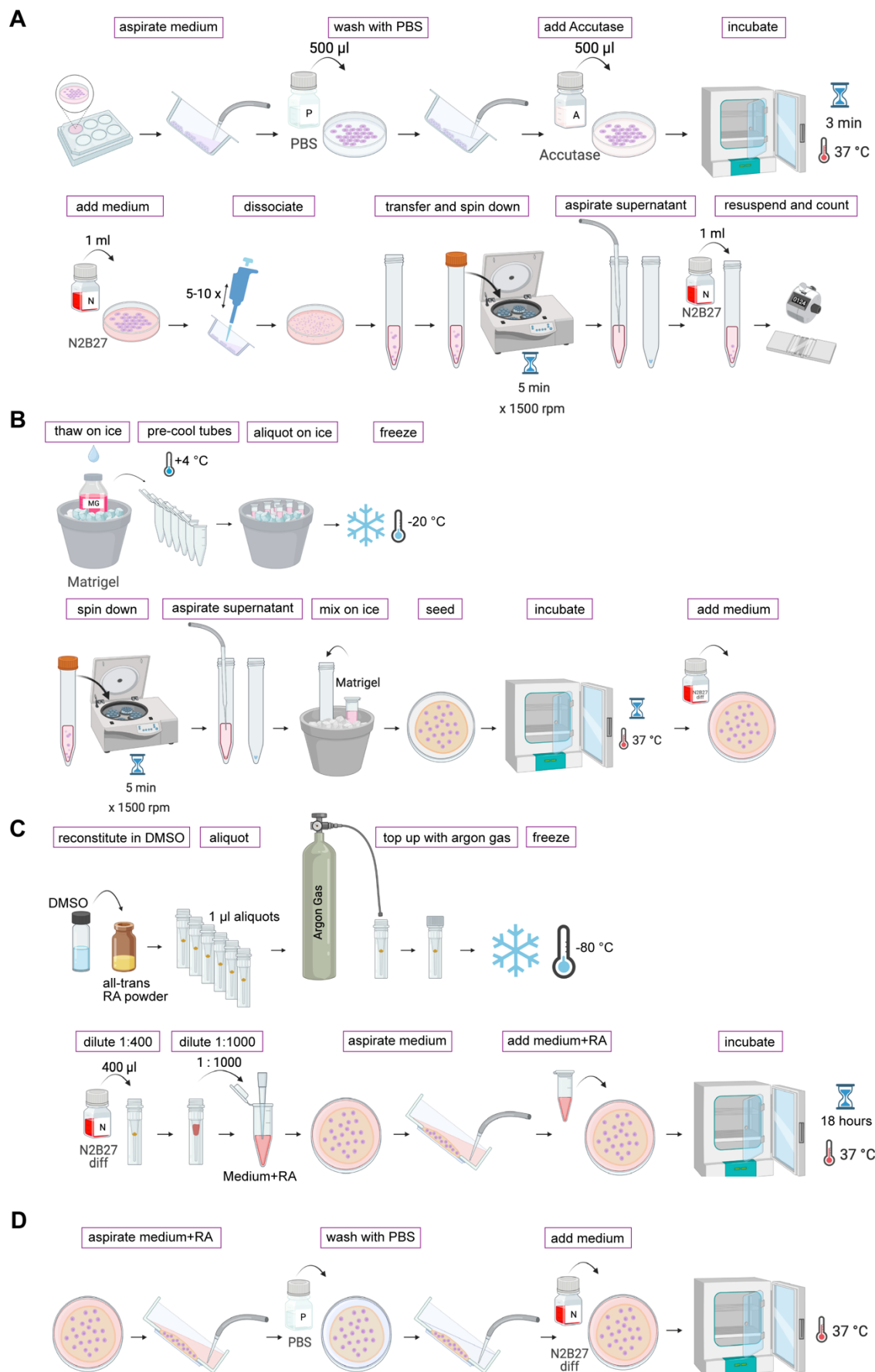


**Figure 1: Overview of the protocol.**

**A:** Passaging ESCs (see 1.1.).

**B:** Aliquoting Matrigel and seeding single ESCs for differentiation into NTOs (see 1.2.).

**C:** Aliquoting RA and RA treatment (see 2.).

**D:** RA removal and routine of daily medium replacement (see 3.).

This figure was created with [BioRender.com](https://BioRender.com)

## Figure 2

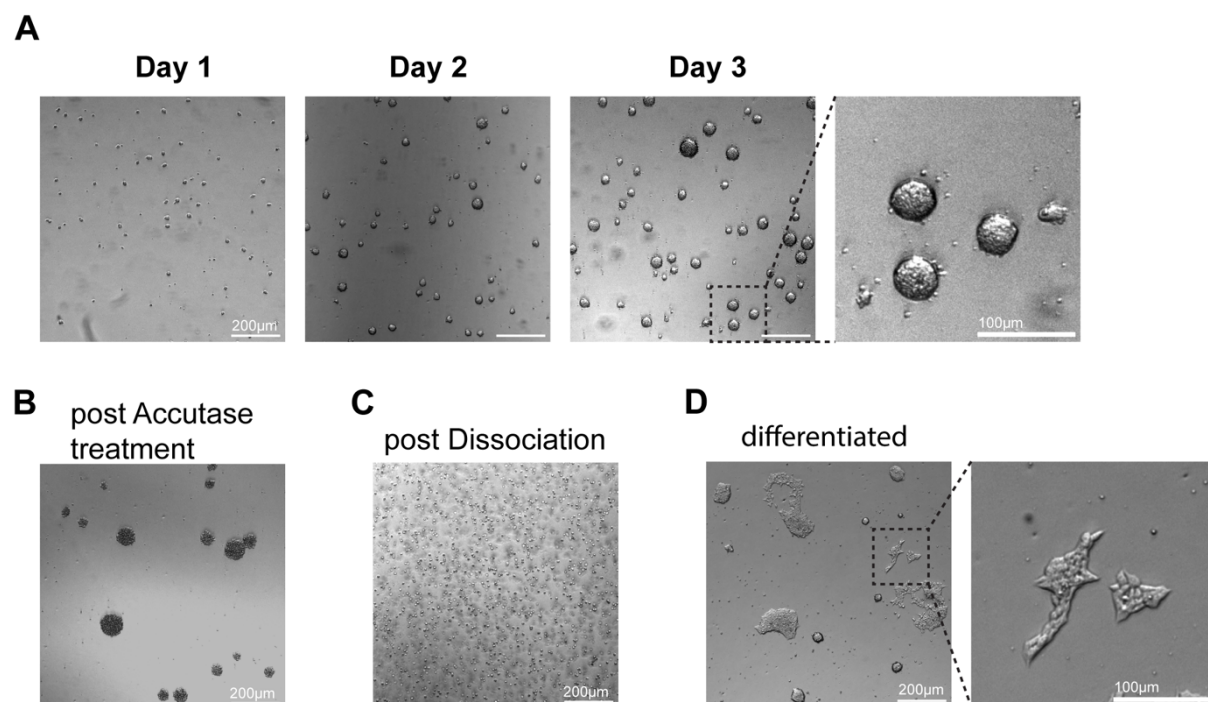


**Figure 2: Good quality mouse ESCs (R1) maintained in N2B27 supplemented with 2iLIF ('2iLIF' in this protocol).**

**A:** Brightfield images of mouse ESCs 1-3 days after passaging. Colonies are doom-shaped and have a round, smooth outline. Scale bars, 200  $\mu\text{m}$  (4x image) and 100  $\mu\text{m}$  (zoom)

**B:** Day 3 ESCs after 3 minutes of Accutase treatment at 37  $^{\circ}\text{C}$ . Colonies are detached from the bottom of the culture dish and single cells can be observed within the colony. Scale bar, 200  $\mu\text{m}$

**C:** Colonies from B after dissociation into single cells. Scale bar, 200  $\mu\text{m}$

**D:** Differentiated ESCs with colonies looking jagged (spikey edges) and flat. Scale bars, 200  $\mu\text{m}$  (4x image) and 100  $\mu\text{m}$  (zoom)

**Figure 3**

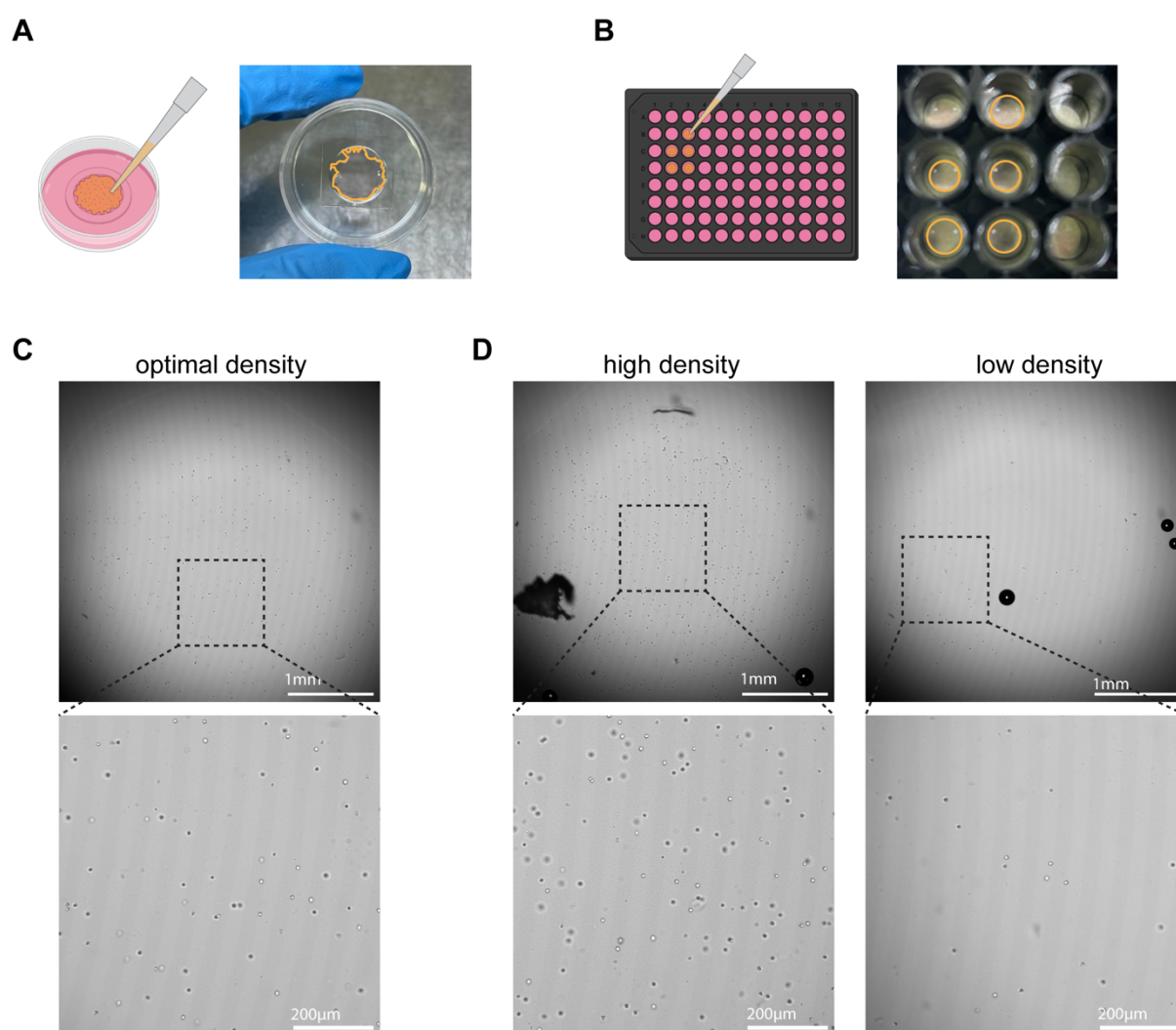


**Figure 3: Seeding density in Matrigel in MatTek dish (A) and 96-well plate (B).**

**A:** Schematic of the Matrigel-cell suspension seeding process (orange) in a 35 mm glass bottom MatTek dish. Suspension is seeded using 'flower' technique to avoid majority of the cells being seeded close to the walls.

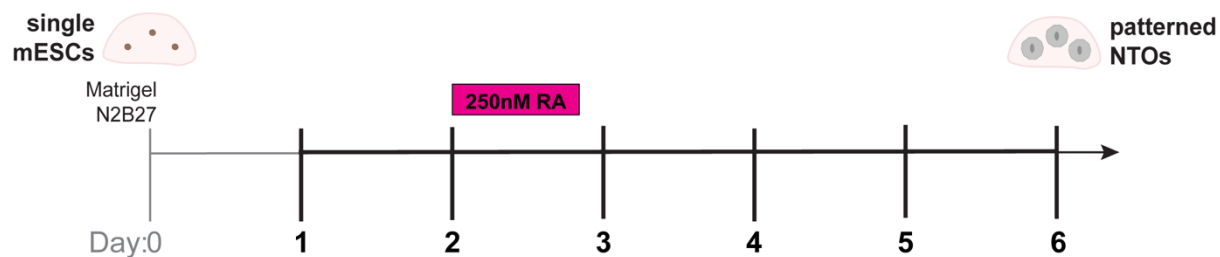
**B:** Schematic of the Matrigel-cell suspension seeding process (orange) in a well of a 96-well plate. Suspension is seeded in a circle to avoid majority of the cells being seeded close to the walls.

**C:** Optimal seeding density of 100 cells/ μl Matrigel (Table 2). Scale bars, 1 mm (4x image) and 200 μm (zoom)

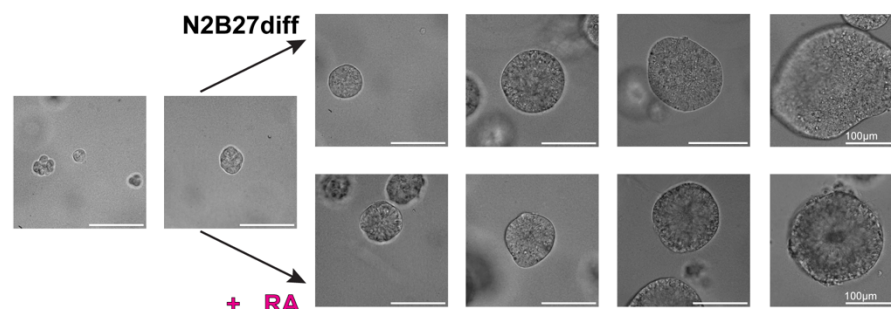
**D:** Suboptimal seeding density. Left panel: cell density too high (1000 cells/ μl Matrigel), right panel: cell density too low (10 cells/ μl Matrigel). Scale bars, 1 mm (4x image) and 200 μm (zoom)

## Figure 4

**A**

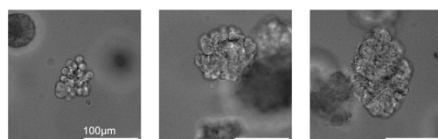


**B**



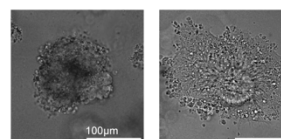
**C**

failed/ delayed epithelialization



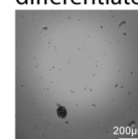
**D**

dying/ stressed NTOs



**E**

no NTOs from differentiated ESCs



## Figure 4: representative NTOs.

**A:** Schematic of NTO differentiation protocol with 18 hours 250 nM RA pulse from day 2.

**B:** Representative results of NTOs from day 1-6 according to timeline in A in N2B27diff (upper panel) or + RA (lower panel). Scale bars, 100 μm

**C:** Example NTOs showing failed or delayed epithelialization. Scale bars, 100 μm

**D:** Example NTOs showing dying or stressed NTOs. Scale bars, 100 μm

**E:** Representation of day 6 'NTOs' from differentiated ESCs. Scale bar, 200 μm

**Figure 5**

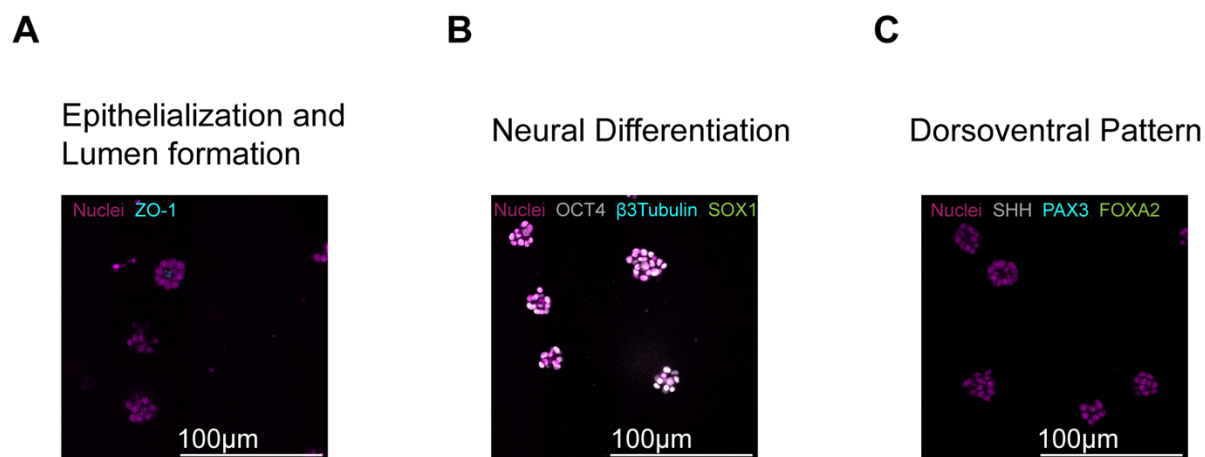


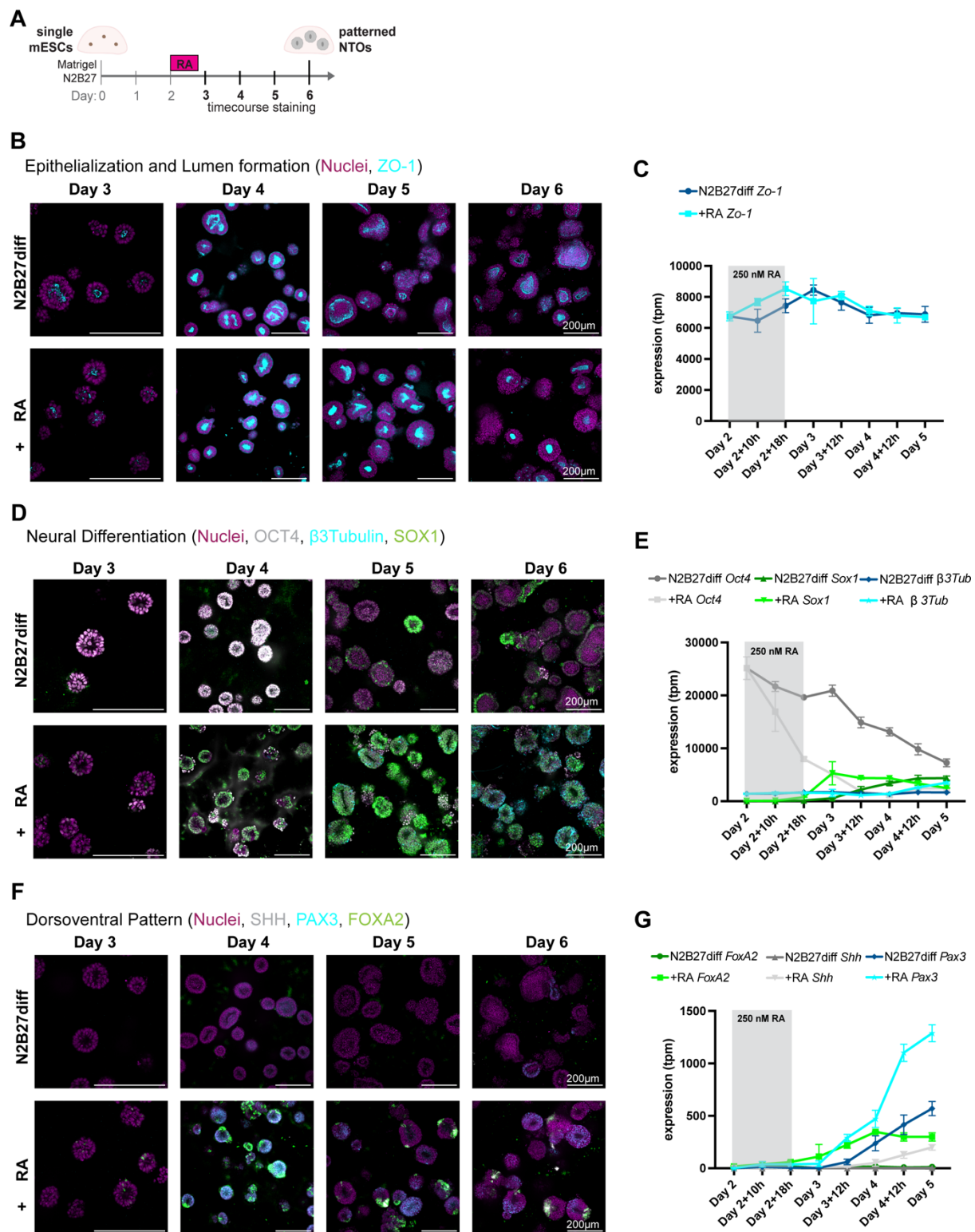
**Figure 5: Marker expression in day 2 NTOs before RA treatment.**

**A:** Representative NTOs for epithelialization and lumen formation, with nuclei (magenta) and ZO-1 (cyan). Scale bars, 100 μm

**B:** Representative NTOs for neural differentiation, with nuclei (magenta), OCT4 (grey), β3Tubulin (cyan) and SOX1 (green). Scale bars, 100 μm

**C:** Representative NTOs for dorsoventral pattern, with nuclei (magenta), SHH (grey), PAX3 (cyan) and FOXA2 (green). Scale bars, 100 μm

**Figure 6**



# **Figure 6: Marker expression from day 3-6 in control (N2B27diff) and RA-treated (+ RA) NTOs.**

**A:** Schematic of the NTO differentiation assay with 18 hours 250 nM RA pulse from day 2 and timecourse staining from day 3-6.

**B:** Representative timecourse of NTOs for epithelialization and lumen formation, with nuclei (magenta) and ZO-1 (cyan). Scale bars, 200  $\mu$ m

**C:** Timecourse RNA sequencing<sup>25</sup> for *Zo-1* (dark blue respectively cyan) from day 2-5 in N2B27diff and + RA.

**D:** Representative timecourse of NTOs for neural differentiation, with nuclei (magenta), OCT4 (grey),  $\beta$ 3Tubulin (cyan) and SOX1 (green). Scale bars, 100  $\mu$ m

**E:** Timecourse RNA sequencing<sup>25</sup> for *Oct4* (dark respectively light grey), *Sox1* (dark respectively light green) and  *$\beta$ 3Tubulin* (dark blue respectively cyan) from day 2-5 in N2B27diff and + RA.

**F:** Representative timecourse of NTOs for dorsoventral pattern, with nuclei (magenta), SHH (grey), PAX3 (cyan) and FOXA2 (green). Scale bars, 200  $\mu$ m

**G:** Timecourse RNA sequencing<sup>25</sup> for *Shh* (dark respectively light grey), *Pax3* (dark blue respectively cyan) and *FoxA2* (dark respectively light green) from day 2-5 in N2B27diff and + RA.

| N2B27 base (for 50 ml) |                          | 2iLIF (for 50 ml) |            | N2B27 diff (for 50 ml) |                 |
|------------------------|--------------------------|-------------------|------------|------------------------|-----------------|
| 25 ml                  | Neurobasal               | 50 ml             | N2B27 base | 50 ml                  | N2B27 base      |
| 25 ml                  | DMEM/ F12                | 20 ng/ml          | mouse LIF  | 0.5 ml                 | Sodium Pyruvate |
| 0.5 ml                 | B27                      | 3 $\mu$ M         | CHIR 99021 |                        |                 |
| 0.25 ml                | N2                       | 1 $\mu$ M         | PD 0325901 |                        |                 |
| 0.5 ml                 | Pen/ Strep               |                   |            |                        |                 |
| 0.5 ml                 | Glutamine                |                   |            |                        |                 |
| 0.1 mM                 | $\beta$ -Mercaptoethanol |                   |            |                        |                 |

**Table 1:** Medium components (N2B27 base, 2iLIF, N2B27diff)

| Dish/ well                      | Matrigel        | Medium/ Volume                        | Cells (at 100 cells/ $\mu$ l Matrigel density) | Jellification time (37 °C) |
|---------------------------------|-----------------|---------------------------------------|------------------------------------------------|----------------------------|
| MatTek (35mm glass bottom dish) | 40 $\mu$ l      | 2 ml                                  | 4000                                           | 15 minutes                 |
| 96-well plate                   | 4 $\mu$ l       | 100 – 200 $\mu$ l (depending on dish) | 400                                            | 4 minutes                  |
| 24-well plate                   | 10 – 15 $\mu$ l | 1 ml                                  | 1000 – 1500                                    | 10 minutes                 |
| 6-well plate                    | 40 – 80 $\mu$ l | 2 ml                                  | 4000 – 8000                                    | 15 minutes                 |

**Table 2:** Information on cell number per dish, amount of Matrigel, volume of medium and jellification time.

## DISCUSSION:

Here, we present several optimizing adaptations and improvements to the original protocol for generating high quality NTOs, to facilitate standardization across laboratories. Key changes include different ESC culture conditions, reduced input cell numbers, improved differentiation medium, modified differentiation protocol, and seeding density adjustments for high-throughput analysis using multi-well plates.

The most critical points of the protocol are:

- Source and use of the correct Matrigel with appropriate protein concentration (inappropriate protein concentration can affect NTO patterning efficiency, see Table 3)
- Seeding of single cells in the correct density (seeding clumps of cells can lead to 'grape'-formation and non-epithelialized NTOs with multiple lumen <sup>25</sup>)
- Complete removal of 2iLIF from ESC maintenance medium before seeding for NTOs (carry-over of 2iLIF can impair NTO epithelialization, see Table 3)
- RA treatment using 250 nM for 18 hours on day 2 is crucial for pattern formation as tested in <sup>17</sup> (lower concentration and/or shorter pulse decreases pattern efficiency)
- Fixation with 2% PFA (using higher concentrated PFA can dissolve Matrigel)
- Prolonged staining combined with overnight antibody incubation to ensure full penetration that allows for subsequent 3D analysis (shorter incubation time can cause incomplete penetration and thus impair downstream analysis)
- Correct refractive index and pH of the clearing solution, and appropriate time of addition (only after antibody staining and shortly before imaging) (for structures >200µm 30-60 minutes prior to imaging are sufficient)

Furthermore, establishing a good quality and homogenously pluripotent ESC culture <sup>21</sup> is crucial for the culture's successful and reproducible differentiation into NTOs. Using 'suboptimal' quality cells leads to marked inconsistencies in data acquired, sacrificing time and resources. Differentiated ESCs will not form NTOs and die as single cells in Matrigel. The following points are critical and need to be checked precisely to culture good quality ESCs:

- Culture conditions: ideally, 50 000 cells are seeded per passage, in a well of a 6-well plate with passaging every 2-3 days and medium replacement daily from day 2. Inconsistency in seeding density and irregularity in medium replacement

can have severe negative effects on the quality of the cells (see Table 3 for more details). We recommend to culture ESCs in 2iLIF medium on 0.15% gelatin-coated plates as this provides the most homogenously pluripotent starting culture <sup>21</sup>.

- Differentiation: differentiated cells will not be forming NTOs but remain single cells in the Matrigel and die (which results in lower number of 'good' NTOs, see Table 3).
- Contamination: Mycoplasma contamination delays NTO formation and growth, resulting in smaller, stressed organoids that eventually die. Neural differentiation and patterning efficiency are also severely affected when ESCs contaminated with mycoplasma.

| Problem                                               | Possible reason                                     | Solution                                                                                                                                                                                                                                                               |
|-------------------------------------------------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cells do not form NTOs (single cells in Matrigel die) | Toxic medium                                        | Check if all reagents are fresh and not expired, if necessary, test all medium components by replacing them individually with new/ fresh ones                                                                                                                          |
|                                                       | Detergent in the dish                               | If the dish was re-used, wash carefully 3x with sterile water, then 3x with EtOH, then let dry and UV for 1 hour. or: discard and open fresh bag of dishes/ different batch/ lot number                                                                                |
|                                                       | ESCs were differentiated                            | If ESCs were differentiated, this can be tested by 1) antibody staining for pluripotency markers, 2) visual assessment of cell shape.<br>In that case: discard and thaw fresh ESCs                                                                                     |
| Delayed/ failed epithelialization                     | ESCs medium factors (2iLIF) not washed out properly | To avoid 2iLIF carry-over, always wash with PBS when planning to seed NTOs (one wash before Accutase treatment and ESC clump dissociation should be fine, but there can be another washing step included after counting the ESCs before resuspending them in Matrigel) |
|                                                       | Old differentiation medium (>1-2 weeks) used        | Do not use N2B27 that is older than 2 weeks. We recommend preparing fresh N2B27diff on the day or a day before seeding for NTOs, because an assay will take around 7 days                                                                                              |

|                      |                                                                                       |                                                                                                                                                                                                                                                                                                                                                  |
|----------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                      | Wrong components of N2B27diff (DMEM/F12 + Glutamine/ Glutamax, HEPES, B27 -VitA, ...) | It is crucial to use the correct medium components and concentrations, as wrong concentrations (of e.g. glutamine) delay/ hinder epithelialization which impacts the efficacy of the assay (check labels, as well as catalogue and lot numbers)                                                                                                  |
|                      | Too much Glutamine/ Glutamax                                                          | See above: when too much Glutamine (or Glutamax (more stable)) is added to the medium, the differentiation assay is unreliable. Only use the recommended concentration of Glutamine, added fresh to the medium on the day of preparation.                                                                                                        |
|                      | Suboptimal ESC condition                                                              | If ESCs are not entirely pluripotent, this can delay/ hinder epithelialization (discard ESCs and thaw fresh ESCs)                                                                                                                                                                                                                                |
|                      | Suboptimal pH of medium                                                               | If the pH is slightly too acidic, this can delay epithelialization of NTOs can be delayed. Keeping the medium in the fridge for 2-3 days before seeding for differentiation assay helps acquire the right pH; check that there is no HEPES in the medium because HEPES buffers the pH and will hinder the appropriate epithelialization of NTOs. |
|                      | CO <sub>2</sub> suboptimal                                                            | Suboptimal CO <sub>2</sub> levels can severely impact epithelialization and growth of NTOs (check/ service incubators on a regular basis)                                                                                                                                                                                                        |
| Dying/ stressed NTOs | Suboptimal storage of RA                                                              | RA is sensitive to light and air. If RA was stored under suboptimal conditions, discard the batch and reconstitute fresh from powder                                                                                                                                                                                                             |
|                      | Suboptimal ESC condition                                                              | Differentiated ESCs do not result in adequate NTOs if any are formed (depending on the degree of differentiation). Discard and thaw fresh ESCs                                                                                                                                                                                                   |
|                      | NTOs dried during medium replacement                                                  | If cells in Matrigel dried during any of the protocol steps, NTOs will be stressed and eventually die. Proceed with removing 'old' medium in the wells/ dishes only once fresh                                                                                                                                                                   |

|                                     |                                                        |                                                                                                                                                                                                                                                         |
|-------------------------------------|--------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                     | Medium not replaced daily                              | medium is already prepared and ready to use (pre-warmed).                                                                                                                                                                                               |
|                                     | Old differentiation medium (>1-2 weeks) used           | NTOs that lack nutrients because medium was not replaced daily will get stressed and die. Replace medium daily from day 2 onwards.                                                                                                                      |
|                                     | Expired medium components used                         | Recommended: always prepare fresh N2B27diff on the day or a day before seeding NTOs.                                                                                                                                                                    |
|                                     | Matrigel was thawed for too long                       | Never use expired reagents for NTOs.                                                                                                                                                                                                                    |
|                                     | Suboptimal seeding density (too low or too high)       | Matrigel that was kept for too long at 4°C will turn pink and differ in concentration of growth factors and stiffness. Discard and thaw a fresh aliquot for every differentiation assay (making smaller aliquots is recommended).                       |
|                                     | Suboptimal/ fluctuating temperature (incubator/ bench) | NTOs that were seeded too densely or sparsely will get stressed and eventually die (use optimal seeding density as recommended in Table 2).                                                                                                             |
|                                     | Mycoplasma infection of ESCs                           | Temperature fluctuations cause NTO stress and death. Never keep NTOs out of the incubator for longer than necessary (only for checking under the microscope and changing medium), carry out regular cleaning, service and maintenance of the incubator. |
|                                     | Suboptimal $\beta$ -Mercaptoethanol concentration      | Test for Mycoplasma on a regular basis. If ESCs are infected, discard them and thaw fresh.                                                                                                                                                              |
| (Ventral) patterning efficiency low | low RA concentration                                   | Adhere to the concentrations and volumes present in Table 1.                                                                                                                                                                                            |
|                                     |                                                        | Either the stock was incorrectly reconstituted, or the aliquots were diluted incorrectly. As shown in Meinhardt et al. <sup>17</sup> different concentrations of RA can induce patterning (so this must be vastly off).                                 |

|                                         |                                                         |                                                                                                                                                                                                                                                                            |
|-----------------------------------------|---------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                         | Suboptimal Matrigel lot                                 | If Matrigel protein concentration is much lower than 10 mg/ml, it can diminish patterning efficiency because of its variation in growth factor expression and/or stiffness <sup>26</sup> . Test different lots of Matrigel with higher protein concentration if necessary. |
|                                         | Suboptimal seeding density (too low or too high)        | Only the optimal seeding density leads to 80% patterned NTOs by day 6. Best to adhere to proposed cell numbers as mentioned in Table 2.                                                                                                                                    |
|                                         | Suboptimal ESC condition                                | Stressed ESCs (lack of nutrients, wrong density, overgrown, etc.) will not pattern as efficiently.                                                                                                                                                                         |
| Late expression of pluripotency markers | Carry-over of 2iLIF components in differentiation assay | To avoid carry over of ESC medium, always wash with PBS before seeding for differentiation assay.                                                                                                                                                                          |
|                                         | Too much Glutamine/ Glutamax                            | Using the appropriate amount of Glutamine/ Glutamax is crucial, check recipe precisely.                                                                                                                                                                                    |
|                                         | Cell lines used                                         | Cell lines of different origin might slightly differ in their neural differentiation timing.                                                                                                                                                                               |

**Table 3: Troubleshooting of the method.**

This protocol has been rigorously tested using the cell lines R1<sup>27</sup>, 46C<sup>28, 29</sup>, IB10<sup>30</sup>, ES-E14TG2a<sup>31–33</sup>, 129S6<sup>34</sup>, HM1<sup>33</sup>, and we confirmed that they feasibly made NTOs. Other cell lines (e.g. with C57BL/6 background), might require optimizations. Slight differences in anterior-posterior identity, dorsoventral patterning efficiency, and degree of ventralization can be observed among different cell lines. We acknowledge the presence of variability and heterogeneity in organoid size, despite starting from single cells. Because Matrigel is an expensive, animal-derived resource that is often sparsely available and has unpredictable delivery times, PEG-based hydrogels can be used alternatively <sup>26</sup>.

This protocol was optimized from Meinhardt et al.<sup>17, 20</sup> to increase survival of ESCs in Matrigel, as well as epithelialization and patterning efficiency of NTOs.

- Defined, more homogenous pluripotent ESC culture.

- Lower cell number required (due to homogeneous pluripotent starting culture, more single cells survive and form NTOs).
- Increased patterning efficiency (from approx. 40% (Meinhardt et al.) to 80%) due to factors mentioned above together with updated differentiation medium formulation (new in this protocol).
- No Noggin supplementation is required in the initial two days before the RA pulse.
- High throughput analysis possible due to adaption to multi-well usage.

To our knowledge, this neural organoid protocol uniquely enables progeny from a single cell to spontaneously undergo patterning upon self-organization following a global pulse of RA in a defined medium, without the need for additional factors or microfluidics to mimic spatial stimulus gradients.

NTOs can be used to study aspects of floorplate self-organization such as reported by Krammer et al. 2024<sup>19</sup>. This work demonstrates the formation of organizers, and it challenges the previous notion that embryonic induction relies solely on a controlling structure, such as the notochord. Instead, it reveals that these processes can occur through the regulated interconnections among cells.

Studying self-organization in an organoid system can give us novel insights into patterning in the developing organism. By manipulating various factors during NTO formation, epithelialization or patterning, key regulators of neural tube development, the underlying mechanisms of certain developmental disorders and potential targets for intervention can be identified. Furthermore, NTOs offer a platform for drug screening and development, where the efficacy and safety of potential therapeutic compounds can be tested in a more physiologically relevant environment.

This protocol is a standard in the Tanaka laboratory and highlights the versatility of NTOs and their potential to advance various areas of neuroscience, developmental biology, and regenerative medicine.

## TABLE OF MATERIALS:

| Name of Material/ Equipment                                      | Company                             | Catalog Number                | Comments/ Description          |
|------------------------------------------------------------------|-------------------------------------|-------------------------------|--------------------------------|
| 35 mm Dish   No. 1.5 Coverslip   14 mm Glass Diameter   Uncoated | MatTek Corporation                  | Cat# P35G-1.5-14-C            |                                |
| Accutase                                                         | Invitrogen                          | Cat# 00-4555-56               |                                |
| Albumin (BSA) Fraction V (pH 7.0)                                | Panreac AppliChem                   | Cat# A1391                    |                                |
| All-Trans Retinoic Acid                                          | Merck                               | Cat# R2625                    |                                |
| B-27 Supplement (50X), serum free                                | Gibco                               | Cat# 17504044                 |                                |
| Basement Membrane Matrix (Matrigel)                              | Corning                             | Cat# BDL354234                | Protein concentration 10 mg/ml |
| CellCarrier-96 Ultra Microplates                                 | PerkinElmer                         | Cat# 6055302                  |                                |
| CHIR 99021                                                       | Tocris                              | Cat# 4423                     |                                |
| CUBIC-R+(N)                                                      | Matsumoto et al, 2019 <sup>35</sup> | N/A                           |                                |
| Dimethylsulfoxid                                                 | Sigma Aldrich                       | Cat# D2650                    |                                |
| DMEM/F-12, no glutamine                                          | Gibco                               | Cat# 21331020                 |                                |
| Dulbecco's Phosphate-buffered saline (DPBS, 1X)                  | Gibco                               | Cat# 14190144                 |                                |
| Gelatin                                                          | Merck                               | Cat# G2500                    |                                |
| L-Glutamine (200 mM)                                             | Gibco                               | Cat# A2916801                 |                                |
| 2-Mercaptoethanol (50 mM)                                        | Gibco                               | Cat# 31350010                 |                                |
| Monoclonal mouse anti-OCT-3/4                                    | Santa Cruz Biotechnology            | Cat# sc-5279; RRID:AB_628051  | Use 1:200                      |
| Monoclonal mouse anti-PAX3                                       | DSHB                                | Cat# PAX3-C; RRID:AB_528426   | Use 1:100                      |
| Monoclonal rabbit anti-SHH                                       | Cell Signalling Technologies        | Cat# 2207; RRID:AB_2188191    | Use 1:300                      |
| Monoclonal rabbit anti-β3-Tubulin                                | Cell Signalling Technologies        | Cat# 5568; RRID:AB_10694505   | Use 1:500                      |
| Monoclonal mouse anti-ZO-1                                       | Invitrogen                          | Cat# 33-9100; RRID:AB_2533147 | Use 1:200                      |
| Mouse LIF                                                        | Qkine                               | Cat# Qk018-1000               |                                |
| N-2 Supplement (100X)                                            | Gibco                               | Cat# 17502048                 |                                |
| Neurobasal Medium                                                | Gibco                               | Cat# 21103049                 |                                |
| Paraformaldehyde, reagent grade, crystalline                     | Merck                               | Cat# P6148                    |                                |
| PD 0325901                                                       | Tocris                              | Cat# 4192                     |                                |
| Penicillin-Streptomycin Solution                                 | Gibco                               | Cat# 15140130                 |                                |
| Polyclonal goat anti-FOXA2                                       | R&D                                 | Cat# AF2400; RRID:AB_2294104  | Use 1:400                      |
| Polyclonal goat anti-SOX1                                        | R&D Systems                         | Cat# AF3369; RRID:AB_2239879  | Use 1:200                      |
| RNA-Seq data related to Figure 6                                 | Ishihara et al., 2022 <sup>25</sup> | GEO: GSE214368                |                                |
| Sodium Pyruvate (100 mM)                                         | Gibco                               | Cat# 11360070                 |                                |
| Triton X-100                                                     | Sigma Aldrich                       | Cat# 9036-19-5                |                                |

## **ACKNOWLEDGMENTS:**

We would like to thank present and past members of the Tanaka lab for their support and discussions in the development of this protocol. Especially, we thank A. Pelzl for critically reading the manuscript, giving input and helping with schematics, and to E. Chatzidaki for support. This publication was funded by the Austrian Science Fund (FWF): “Stand Alone publication project number PUD 25”. Additionally, this project has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement ERC AdG 742046), and from the Austrian Science Fund (FWF) [10.55776/ F7803-B] (Stem Cell Modulation). For the purpose of Open Access, the authors have applied a CC BY public copyright license to any Author Accepted Manuscript (AAM) version arising from this submission.

## **DISCLOSURES:**

The authors declare no conflict of interest.

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## III.2 “Mouse neural tube organoids self-organize floorplate through BMP-mediated cluster competition”

Neural tube organoids (NTOs) formed from clonal mouse embryonic stem cells (ESCs) structurally and functionally resemble the early neural tube *in vivo*. However, in contrast to its *in vivo* equivalent, NTOs form a localized signaling center, the floorplate, without its embryonic inducer the notochord, through the process of self-organization. In this manuscript, we investigated the mechanism underlying this organizer self-organization after a global pulse of retinoic acid (RA). We found that initially dispersed FOXA2<sup>+</sup> floorplate cells form intermediate clusters that compete over long distances within the organoid to establish the final floorplate. We further showed that this long-range cluster competition is mediated by diffusible molecules of the BMP pathway.

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| <b>Status:</b>        | Published research article in the journal<br>Developmental Cell<br>Publication date: 21.05.2024                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| <b>Authors:</b>       | <u><b>Teresa Krammer*</b></u> , Hannah T Stuart*, Elena Gromberg*, Keisuke Ishihara, Dillon Ciso, Manuela Melchionda, Fernando Becerril Perez, Jingkui Wang, Elena Costantini, Stefanie Lehr, Laura Arbanas, Alexandra Hörmann, Ralph A Neumüller, Nicola Elvassore, Eric Siggia, James Briscoe, Anna Kicheva, Elly M Tanaka<br>* equal contribution                                                                                                                                                                                                                                                                                                                                                                                                             |
| <b>Contributions:</b> | <u><b>T.K.</b></u> , H.T.S., and E.G. designed and performed experiments, created methodologies, and analyzed the data. K.I. provided supervision, set up live imaging conditions, developed image analysis pipelines, and helped to analyze data. D.C. and E.S. developed image analysis pipelines. M.M. and J.B. generated Shh <sup>-/-</sup> ESCs. F.B.P. performed and bioinformatically analyzed BRB sequencing. J.W. performed bioinformatic analyses. E.C. and L.A. performed experiments. S.L. and A.K. provided Nog cKO embryos. A.H. and R.A.N. assisted with the pharmacological screen. N.E. supported the development of larger NTO media. <u><b>T.K.</b></u> , H.T.S., and E.M.T. wrote the manuscript. E.M.T. conceived and supervised the study. |
| <b>DOI:</b>           | 10.1016/j.devcel.2024.04.021                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

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**Neural tube organoids self-organise floorplate through BMP-mediated cluster competition**

Teresa Katharina Krammer; Hannah Taylor Stuart; Elena Gromberg; Keisuke Ishihara; Manuela Melchionda; Jingkui Wang; Elena Costantini; Stefanie Lehr; Dillon Cisko; Laura Arbanas; Alexandra Hörmann; Ralph A Neumüller; Nicola Elvassore; Eric Siggia; James Briscoe; Anna Kicheva; Elly Margaret Tanaka

Our reference: DEVCEL 5904

Article reference: DEVCEL\_DEVELOPMENTAL-CELL-D-23-00771

Article title: Mouse neural tube organoids self-organise floorplate through BMP-mediated cluster competition

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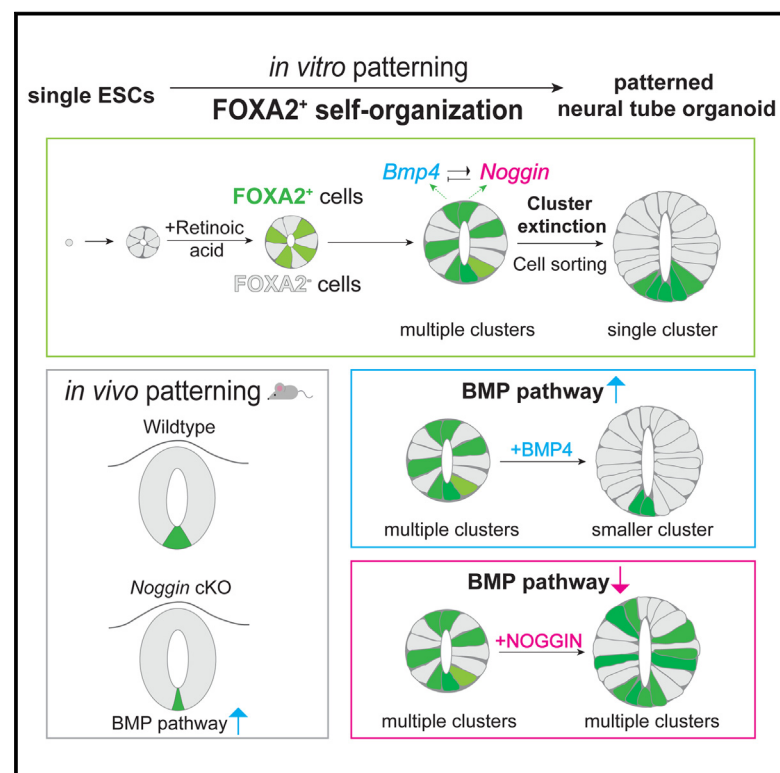
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# Developmental Cell

## Mouse neural tube organoids self-organize floorplate through BMP-mediated cluster competition

### Graphical abstract



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### In brief

Krammer et al. show that retinoic-acid-triggered self-organization of neural tube organoids occurs via induction of scattered FOXA2-expressing cells that undergo clustering and competition to persist and form floorplates. Cluster competition occurs via BMP signaling, which controls floorplate number and size *in vitro* and size *in vivo* at the mid/hindbrain region.

### Highlights

- During neural tube organoid self-organization, scattered FOXA2<sup>+</sup> cells form clusters
- FOXA2<sup>+</sup> clusters compete via long-range signaling to establish a "winning" floorplate
- BMP4 and NOGGIN control numbers and sizes of FOXA2<sup>+</sup> clusters
- NOGGIN is functionally involved in controlling floorplate size *in vivo*

Article

# Mouse neural tube organoids self-organize floorplate through BMP-mediated cluster competition

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<https://doi.org/10.1016/j.devcel.2024.04.021>

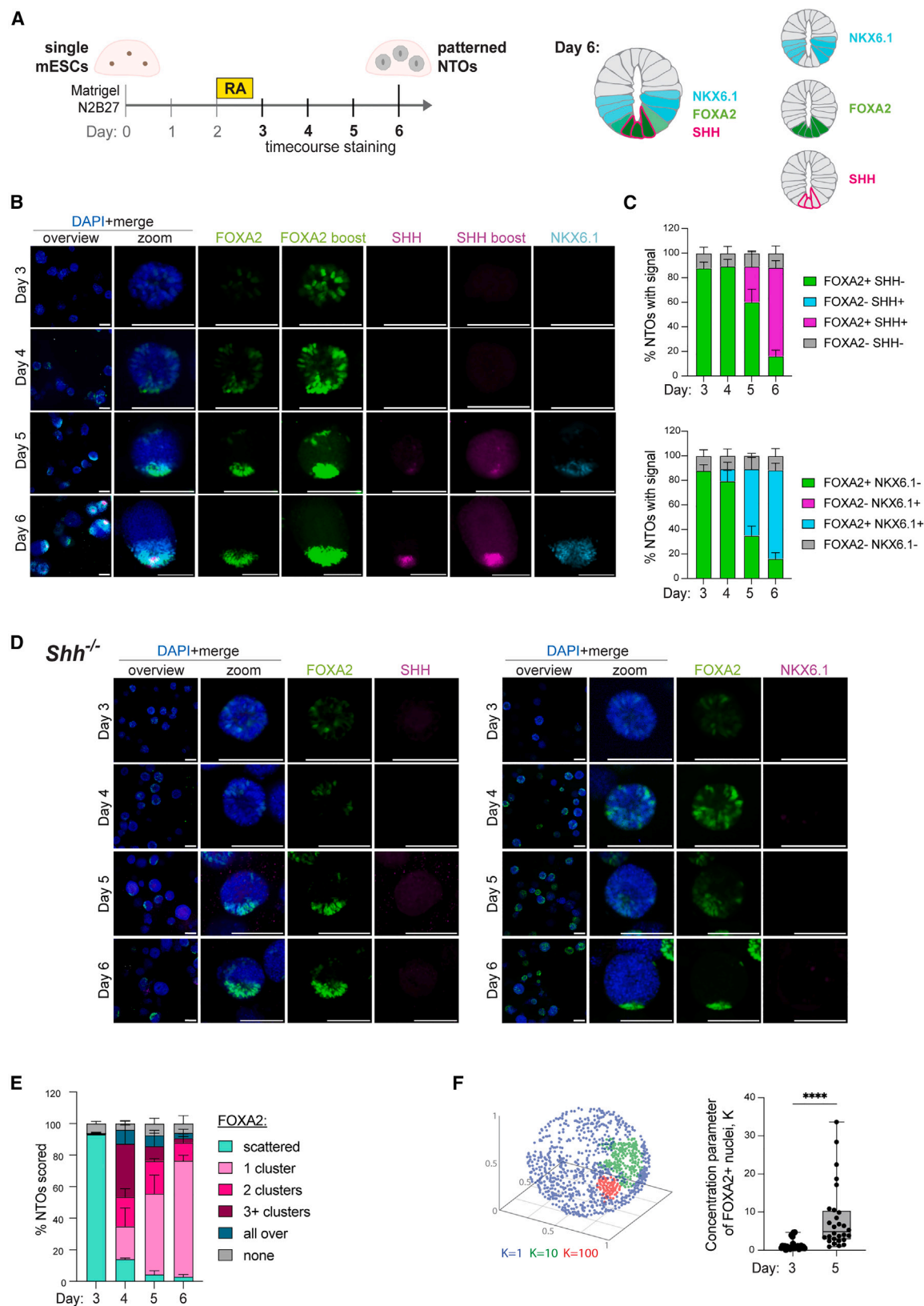
## SUMMARY

During neural tube (NT) development, the notochord induces an organizer, the floorplate, which secretes Sonic Hedgehog (SHH) to pattern neural progenitors. Conversely, NT organoids (NTOs) from embryonic stem cells (ESCs) spontaneously form floorplates without the notochord, demonstrating that stem cells can self-organize without embryonic inducers. Here, we investigated floorplate self-organization in clonal mouse NTOs. Expression of the floorplate marker FOXA2 was initially spatially scattered before resolving into multiple clusters, which underwent competition and sorting, resulting in a stable winning floorplate. We identified that BMP signaling governed long-range cluster competition. FOXA2<sup>+</sup> clusters expressed BMP4, suppressing FOXA2 in receiving cells while simultaneously expressing the BMP-inhibitor NOGGIN, promoting cluster persistence. *Noggin* mutation perturbed floorplate formation in NTOs and in the NT *in vivo* at mid/hindbrain regions, demonstrating how the floorplate can form autonomously without the notochord. Identifying the pathways governing organizer self-organization is critical for harnessing the developmental plasticity of stem cells in tissue engineering.

## INTRODUCTION

During vertebrate development, groups of cells called organizers secrete morphogens to induce cell fate patterning in the surrounding tissues.<sup>1,2</sup> In the embryo, organizers are induced by interactions between emerging tissues (reviewed in<sup>3</sup>). Correct organizer placement relies on pre-existing asymmetries in the embryo and the delivery of polarizing cues. Intriguingly, recent discoveries *in vitro* have found that organizers can form by a process of self-organization in the absence of their natural developmental inducers.<sup>4–7</sup> Despite their central importance for multicellular development as well as tissue regeneration and engineering, the mechanisms by which organizers self-organize are poorly understood.

The neural tube (NT) is the embryonic precursor of the central nervous system. Patterning of neural progenitor identities along the body axes ensures the formation of the correct diversity and arrangement of neurons.<sup>8,9</sup> In the developing spinal cord, NT patterning along the dorsal-ventral axis is guided by two opposing organizers: the dorsal roofplate, secreting bone morphogenetic proteins (BMPs) and Wingless/Integrated (WNTs), and the ventral floorplate, secreting Sonic Hedgehog (SHH) morphogen.<sup>10–13</sup> *In vivo*, the ventral floorplate organizer is usually induced by SHH and other signals from the underlying notochord,<sup>14,15</sup> which has been most extensively studied at thoracic levels. The more anterior floorplate is induced earlier during amniote gastrulation<sup>14,16,17</sup> and forms independently of notochord signaling by a mechanism that is currently unclear.



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The SHH-expressing floorplate has been shown to self-organize in NT organoids (NTOs) *in vitro*<sup>6</sup> in the absence of notochord or any directional inductive cues. NTOs form clonally, by differentiating single mouse embryonic stem cells (ESCs) embedded in three-dimensional (3D) hydrogels,<sup>6,18</sup> resulting in neuroepithelial cysts with a single apical lumen that exhibit a “default” dorsal midbrain identity. A global pulse of retinoic acid (RA) applied at day 2 for 18 h posteriorizes NTOs to hindbrain levels and triggers self-organization of dorsal-ventral patterning by day 6<sup>6</sup> (Figures 1A and S1A). This phenomenon is characterized by the formation of a localized FOXA2<sup>+</sup>SHH<sup>+</sup> floorplate at the emergent ventral pole, which then drives ventral-to-dorsal patterning of neural progenitor identities. Here, we address the mechanism governing the self-organization of this floorplate organizer in NTOs.

## RESULTS

### FOXA2<sup>+</sup> floorplate precursors self-organize from a spatially scattered to a clustered state in clonal NTOs

To characterize the spatiotemporal dynamics of how the FOXA2<sup>+</sup>SHH<sup>+</sup> floorplate self-organizes in mouse NTOs, we first performed time course immunofluorescence (IF) staining after RA pulse (Figure 1A). We assayed FOXA2, SHH, and SHH/GLI-responsive NKX6.1 (Figure 1B) and quantified the number of NTOs expressing each marker (Figure 1C). Temporally, FOXA2 expression was observed from day 3 onward in >80% of NTOs prior to detection of SHH or NKX6.1 proteins. From days 5 to 6, the number of NTOs with FOXA2 and detectable SHH/NKX6.1 increased, whereas SHH or NKX6.1 expression was not observed in FOXA2<sup>−</sup> NTOs (Figure 1C), indicating that FOXA2 preceded SHH expression and activity. Accordingly, RNA sequencing (RNA-seq) analysis<sup>19</sup> showed upregulation of the *FoxA2* transcript earlier than *Shh* or *Nkx6.1* (Figure S1B) and confirmed the expected regional identity of the NTOs, consistent with previous findings by Meinhardt et al.<sup>6</sup> IF and RNA-seq both showed an increase in the level of FOXA2 expression over time from day 3 onward (Figures 1B and S1B).

*In vivo*, SHH from the notochord can induce floorplate FOXA2 expression via GLI binding sites found in FOXA2 regulatory

elements.<sup>15,20</sup> Although we observed FOXA2 expression prior to SHH in NTOs (Figures 1B, 1C, S1B, and S1C), we nonetheless asked whether genetic deletion of SHH would abolish FOXA2 induction and cluster formation. NTOs formed from *Shh*<sup>−/−</sup> ESCs showed no detectable SHH or NKX6.1 expression at any time point (Figures 1D and S1C). However, FOXA2 induction and cluster formation still occurred in *Shh*<sup>−/−</sup> NTOs (Figures 1D and S1D). Unlike parental wild-type (WT) NTOs, the proportion of *Shh*<sup>−/−</sup> NTOs exhibiting FOXA2 expression decreased from 80% to 60% over days 5–6 (Figure S1C), suggesting a role for SHH in FOXA2 maintenance at later time points, consistent with phenotypes of prolonged SHH antagonism in NTOs<sup>6</sup> and the SHH-driven regulatory architecture at the *FoxA2* locus.<sup>20</sup>

Spatially, NTOs expressed FOXA2 at day 3 in a scattered, salt-and-pepper manner (Figure 1B). Subsequently, the FOXA2 signal became increasingly clustered until, by days 5–6, FOXA2 was found in discrete domains inside which SHH expression commenced to yield a functional floorplate, reflected by induction of NKX6.1 (Figures 1B and 1C). Over time, the proportion of NTOs with scattered FOXA2, versus 3+, 2, or 1 cluster per NTO, shifted until a majority of NTOs contained a single FOXA2<sup>+</sup> cluster (Figure 1E). To quantify the spatial distribution of FOXA2<sup>+</sup> nuclei in 3D, we performed von Mises-Fisher analysis in which a concentration parameter *K* is computed, based on the sum of directional vectors from the NTO center, to describe how tightly the vectors are distributed around their average orientation. This confirmed that FOXA2 expression became increasingly concentrated over time (Figure 1F). Together, this shows that floorplate self-organization in RA-treated NTOs is characterized by “salt-and-pepper” FOXA2 induction, followed by clustering of FOXA2<sup>+</sup> floorplate precursor cells, which occurs prior to and independently of SHH expression.

### Intermediate FOXA2<sup>+</sup> clusters interact via competition and sorting during self-organization

We next sought to understand whether and how individual NTOs progress from scattered to multi-cluster to single floorplates. We generated a FOXA2-Venus fusion reporter at the endogenous locus, together with knockin of H2A-mCherry into the safe-harbor *Rosa26* locus to constitutively label nuclei

### Figure 1. Clonal NTOs transition from a scattered expression of FOXA2<sup>+</sup> cells to a clustered state, which can occur in the absence of SHH

(A) Schematic of NTO formation. Single ESCs form epithelial NTOs within 2 days in Matrigel and N2B27. An 18 h pulse of retinoic acid induces floorplate formation and dorsoventral patterning at the endpoint day 6.  
(B) IF time course of NTOs fixed from days 3 to 6 showing (from left to right, single z slice) a merged overview image at 10× magnification, a representative NTO (zoom) stained for DAPI (blue), and the dorsoventral patterning markers FOXA2 (green), SHH (magenta), and NKX6.1 (cyan). In the second FOXA2 column, FOXA2 images were scaled with lower maximal grayscale value to highlight the early spatial pattern. In the second SHH column, the same metric was applied. Scale bars, 100 μm.  
(C) Quantification of marker expression in (*n* = 528) NTOs over time. Colored bars correspond to staining in (B) (*n* = 192 for day 3, *n* = 158 for day 4, *n* = 134 for day 5, and *n* = 44 for day 6).  
(D) *Shh*<sup>−/−</sup> NTOs undergo FOXA2-cell clustering. Time course of *Shh*<sup>−/−</sup> NTOs fixed from days 3 to 6 showing (from left to right, single z slice) a merged overview image at 10× magnification, a representative NTO (zoom) stained for DAPI (blue), and the dorsoventral patterning markers FOXA2 (green) and SHH or NKX6.1 (magenta). For parental control, see Figure S1. Scale bars, 100 μm.  
(E) FOXA2<sup>+</sup> cell cluster distribution over time. FOXA2 spatial distribution was classified into “scattered,” “1 cluster,” “2 clusters,” “3 or more clusters,” “none” (FOXA2<sup>−</sup>), or “all over” (almost entirely FOXA2<sup>+</sup>). A total of 1,148 NTOs were manually analyzed (*n* = 292 for day 3, *n* = 285 for day 4, *n* = 209 for day 5, and *n* = 362 for day 6).  
(F) FOXA2<sup>+</sup> cell clustering analysis using von Mises-Fisher parameter. Left: schematic illustrating quantitative clustering parameter (*K*, from von Mises-Fisher). Right: von Mises-Fisher analysis of 3D FOXA2<sup>+</sup> cell distribution in day 3 (*n* = 27) and day 5 (*n* = 28) NTOs. By day 5, FOXA2-expressing cells show a higher *K* value. Each dot represents the *K* value of a single NTO. Results were statistically analyzed using a Mann-Whitney test with *p* ≤ 0.0001. Error bars show standard deviation (SD), and asterisks mark statistical significance.  
See also Figure S1.

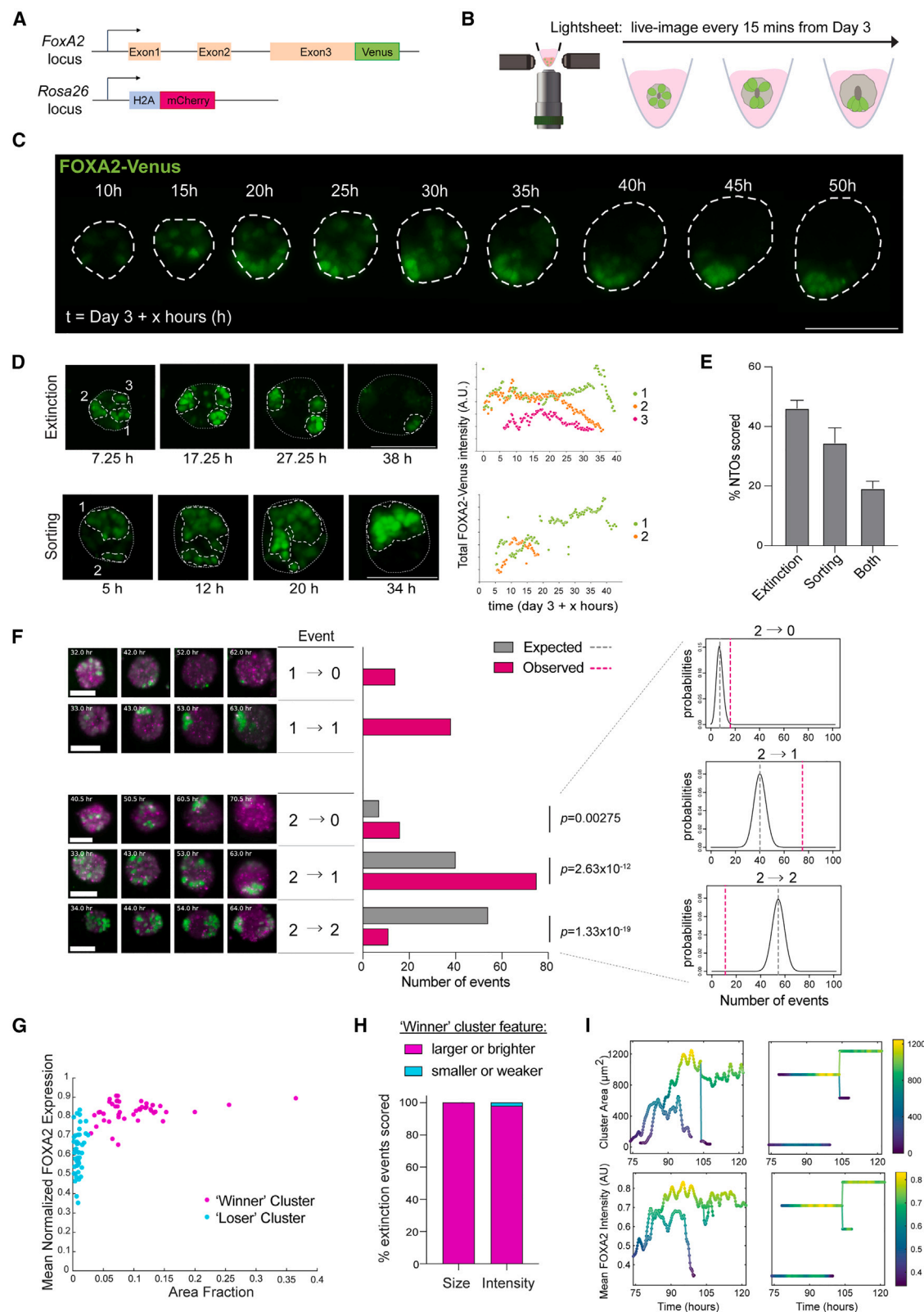


**Figure 2. Statistical analysis and tracking of live cluster dynamics reveal cluster competition during self-organization**

(A) Schematic of double reporter generation. Venus was knocked in after the 3<sup>rd</sup> exon at the C terminus of the endogenous *FoxA2* locus, generating a fusion protein. In addition, an H2A-mCherry construct was knocked into the ubiquitously expressed *Rosa26* locus to mark nuclei.

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(Figures 2A and S2A–S2C). Using 3D light-sheet live imaging, we acquired images every 15 min starting from day 3 in over 300 live NTOs (Figure 2B). This revealed self-organization of FOXA2-Venus<sup>+</sup> cells from a spatially scattered to a clustered state via progressive formation of and resolution between intermediate clusters (Figure 2C; Video S1), in agreement with fixed sample quantifications (Figure 1E).

We observed two distinct modes by which intermediate clusters resolved (Figure 2D; Videos S2 and S3): (1) cluster extinction, whereby FOXA2-Venus expression was lost in some clusters while it persisted in others, and (2) physical cell-sorting, whereby FOXA2-Venus<sup>+</sup> cells from one cluster coalesced with another cluster. These two behaviors were not mutually exclusive: 20% of NTOs exhibited both sorting and extinction of FOXA2-Venus<sup>+</sup> clusters, while 35% and 45% exhibited only sorting or only extinction, respectively (Figure 2E). Because a switch from E- to N-cadherin is observed during neural differentiation from mouse ESCs,<sup>6,18</sup> we examined whether there was differential expression of these cell adhesion molecules between FOXA2<sup>+</sup> and FOXA2<sup>−</sup> cells. We observed E-cadherin expression in FOXA2<sup>+</sup> versus N-cadherin expression in FOXA2<sup>−</sup> cells from day 4 onward, which could explain the sorting behavior (Figures S2D–S2G). Here, however, we focused further on cluster extinction, as it was observed in the majority of NTOs.

To understand whether cluster extinction occurred autonomously or via communication (competition) between clusters, we categorized live-imaged NTOs according to the number of FOXA2-Venus<sup>+</sup> clusters observed at intermediate versus endpoint times. We then tested statistically whether clusters behaved independently of each other. For NTOs with one intermediate cluster, 14 out of 52 extinguished that cluster during self-organization (Figure 2F). By considering the disappearance of a given cluster as a process with this observed probability (0.269), we formulated the null hypothesis that, if FOXA2-Venus<sup>+</sup>

clusters behave independently, the expected outcome for NTOs with two intermediate clusters losing one or both clusters would be 0.393 or 0.072, respectively. We compared these expected outcomes with experimental observations (Figure 2F). In live-imaged NTOs with two intermediate clusters, the observed outcomes differed significantly from the expected outcomes. A cluster was significantly more likely to be extinguished if another cluster was present (Figure 2F), indicating competitive communication between the clusters that governs final outcome.

We aimed to investigate whether there are quantitative features that can predict which cluster will persist and which will be extinguished in competition. To relate cluster features, such as size and FOXA2 intensity, at intermediate time points versus their subsequent outcomes, we established a semi-automated cluster identification pipeline to track FOXA2-Venus<sup>+</sup> clusters over time in live-imaged NTOs in 3D. We performed a detailed analysis on a representative set of movies in which cluster extinction events were observed (20 NTOs comprising 47 extinction events) (Figures 2G–2I, S2H, and S2I; Table S1). When considering the relative properties of clusters in a given NTO 1 h prior to each extinction event, the winning cluster was always larger, occupying >5% of the surface area of the whole NTO (Figures 2G and 2H; Table S1). The winning cluster also exhibited a higher mean FOXA2 intensity in each comparison, except in one case (Figure 2H; Table S1), at the time analyzed. Before that, size and intensity were somewhat predictive of cluster persistence (Figure S2I).

### BMP signaling activity anti-correlates with FOXA2 expression during cluster interaction

We sought to determine the molecular signals that facilitate cluster communication. To identify candidates, we performed bulk RNA-seq of sorted FOXA2-Venus<sup>+</sup> versus FOXA2-Venus<sup>−</sup> cells at day 5, a time point at which cluster extinction was occurring (Figures 3A and S3A). Reasoning that secreted signals could explain

(B) Schematic (created with BioRender) of timelapse imaging of double reporter on the Viventis lightsheet microscope. Full image stacks with 3  $\mu\text{m}$  z step size were taken every 15 min starting 6 h post RA removal on day 3 for 48–75 h.

(C) Images from timelapse series of an NTO (maximum-intensity z-projections), following the spatial organization of FOXA2 (green) over 50 h showing self-organization from a dispersed (salt-and-pepper) into a clustered state (see Video S1). Dashed white lines mark the outlines of the NTO. Scale bars, 100  $\mu\text{m}$ .

(D) Two distinct modes of cluster formation. Selected maximum-intensity z-projection images from two (upper and lower) NTO timelapse series. Upper: “extinction”: three FOXA2<sup>+</sup> (green) clusters form, and then one remains while two disappear (see Video S2). Lower: “sorting”: two distinct clusters grow and coalesce into a single cluster over time (see Video S3). Right: cluster intensities were tracked over time in graphs. Dotted gray lines mark the outlines of NTOs, dashed white lines mark the outline of FOXA2<sup>+</sup> clusters. Scale bars, 100  $\mu\text{m}$ .

(E) A total of  $n = 93$  NTOs from three different timelapses that displayed as 2  $\rightarrow$  1 behavior were subclassified into their mode of self-organization (extinction, sorting, or both), taking into account that sorting might be overestimated due to analysis using maximum-intensity z-projections.

(F) Left: representative time course images for each category, shown as maximum-intensity z-projections; scale bars, 50  $\mu\text{m}$ , and scoring of NTOs according to FOXA2-Venus<sup>+</sup> cluster numbers at intermediate versus endpoint times. Middle: based on the frequency of cluster disappearance in NTOs with one intermediate cluster ( $n = 52$ ), the expected outcomes (gray) for NTOs with 2 intermediate clusters ( $n = 102$ ) were calculated using binomial distribution assuming independent cluster behavior, then compared with observed outcomes (magenta). Right: calculation of  $p$  values based on the binomial distribution for each case, with the expected value indicated by gray lines and the observed numbers by magenta lines.

(G) Measurement of FOXA2 area fraction and mean normalized FOXA2 expression in each cluster, averaged over 1 h time before a cluster was extinguished (“loser” clusters, cyan) versus clusters that persisted (winner clusters, magenta) from movie analyses performed as shown in Figure S2H. A two-sample Kolmogorov-Smirnov (KS) test was used to assess whether the cluster area fraction and the mean normalized cluster FOXA2 expression of the persisting and extinguished clusters were drawn from the same population. This null hypothesis was rejected both for the area fraction data (KS statistic = 1,  $p$  value  $4.7\text{e}^{-22}$ ) and the mean FOXA2 expression data (KS statistic = 0.81,  $p$  value  $1.5\text{e}^{-14}$ ).

(H) For each extinction event, the comparative properties of the persisting winning versus extinguished losing cluster in that NTO are classified.

(I) Topology and features of dynamical FOXA2<sup>+</sup> cluster tracking showing cluster area (upper panel) and mean FOXA2 intensity (lower panel). Results shown are drawn from a single NTO with multiple clusters. Left: dynamical cluster property over time. Right: topology of the directed acyclic graph (DAG) describing the cluster tracking. The y axes of the right plots have no physical meaning and are formatted to provide a clear visual separation between connected components of the DAG.

See also Figure S2 and Videos S1, S2, and S3.

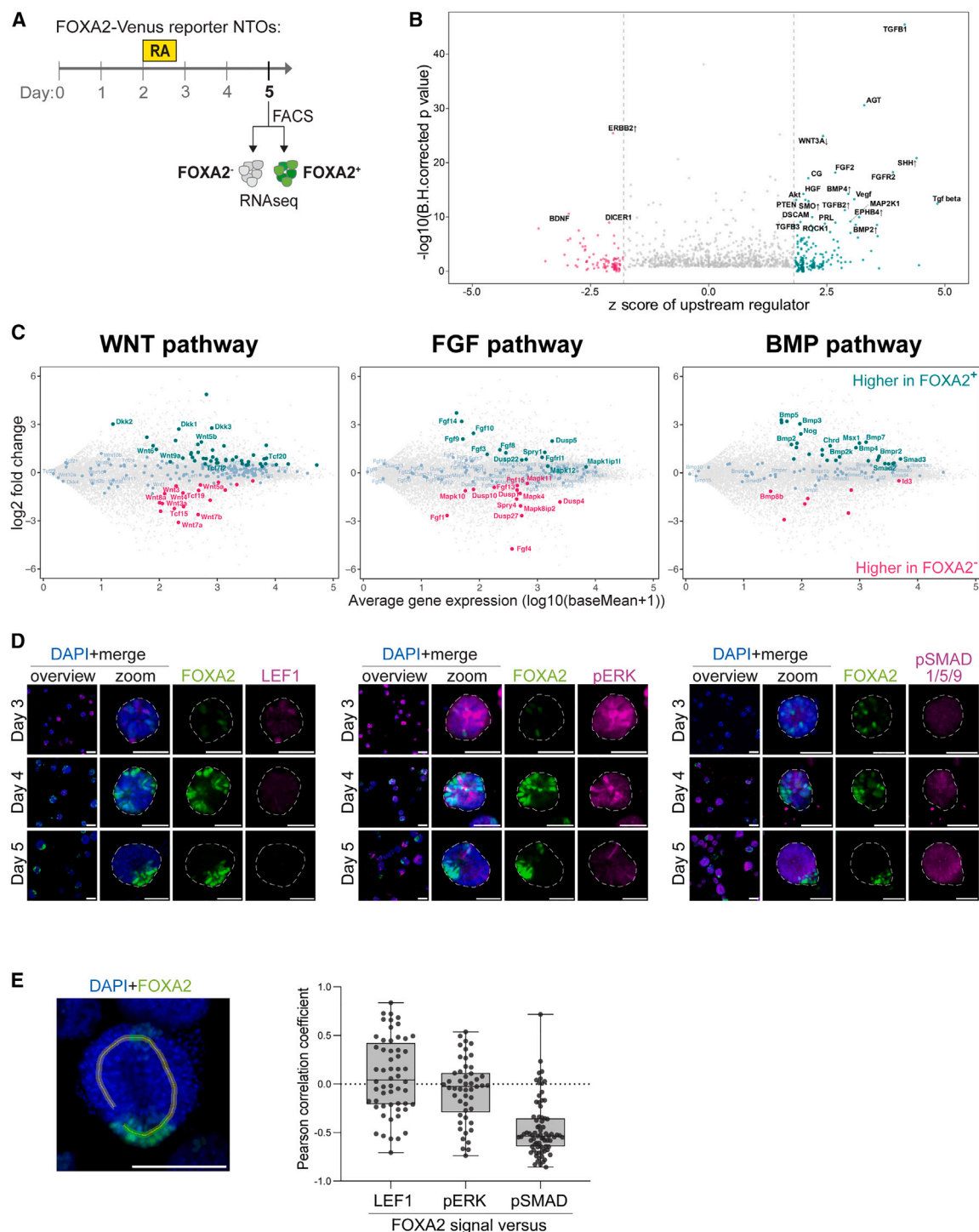


**Figure 3. FOXA2<sup>+</sup> cells express BMPs and *Noggin*, while FOXA2<sup>-</sup> cells display a higher pSMAD1/5/9 signal**

(A) Schematic of RNA-seq performed on sorted FOXA2<sup>+</sup> and FOXA2<sup>-</sup> cell populations of day 5 NTOs made from the reporter cell line.

(B) Activity plot of upstream regulators predicted to be activated or inhibited based on the observed expression changes in FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells. Regulators are colored based on activation Z score at a threshold of 1.8, and the top 20 candidates based on significance are labeled; an arrow next to a regulator name indicates significant up- or downregulation of the gene in FOXA2<sup>+</sup> (versus FOXA2<sup>-</sup>).

(C) WNT, FGF, and BMP pathway members showing differential gene expression between FOXA2<sup>+</sup> and FOXA2<sup>-</sup> cells. MA plot of the RNA-seq data showing log<sub>2</sub> fold changes of genes over their average gene expression against samples (y axis: FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> log ratio values, x axis: log<sub>10</sub>(baseMean + 1) of average gene expression). Color highlights genes assigned to either WNT signaling pathway (GO: 0016055), FGF signaling pathway (GO: 0008543), or BMP signaling

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long-range mutual inhibition between clusters, we focused on the expression of ligands, receptors, transducers, and modulators for the major secreted signaling pathways. Using two methods to test enrichment of signaling pathway signatures in FOXA2<sup>+</sup> versus FOXA2<sup>−</sup> cells (Figures 3B and S3B), signatures of WNT, fibroblast growth factor (FGF), and BMP signaling were uncovered in FOXA2<sup>+</sup> cells. Various WNT and FGF ligands were expressed in FOXA2<sup>+</sup> or FOXA2<sup>−</sup> populations (Figure 3C). Notably, BMP ligands were almost exclusively expressed in FOXA2<sup>+</sup> cells (Figure 3C). Expression of the secreted WNT inhibitors *Dkk1-3* and secreted BMP inhibitors *Noggin* and *Chordin* was enriched in FOXA2<sup>+</sup> cells, whereas FGF-responsive *Spry4* was enriched in FOXA2<sup>−</sup> cells (Figure 3C). We therefore investigated WNT, FGF, and BMP pathways in NTOs by spatial analyses of signaling activities and functional perturbations, as described below.

To functionally test the candidate pathways, we performed pharmacological screens using small molecules and recombinant proteins to activate or inhibit WNT, FGF, or BMP signaling from days 3 to 5 (Figure S3C). WNT or FGF perturbations led to a slight decrease in the percentage of FOXA2<sup>+</sup> NTOs at day 5, irrespective of whether the pathway was activated or inhibited. However, BMP activation decreased—while inhibition increased—the percentage of FOXA2<sup>+</sup> NTOs, demonstrating that the level of BMP signaling modulates FOXA2 expression.

To assess the activities of the WNT, FGF, and BMP pathways during cluster self-organization in NTOs, we assayed for LEF1, phosphorylated (p)ERK, or pSMAD1/5/9, respectively, by IF (Figure 3D). LEF1 and pERK signal decreased, whereas pSMAD1/5/9 increased, from days 3 to 5, suggesting an increase in BMP pathway activity after RA treatment. Interestingly, we observed that the pSMAD1/5/9 signal predominated in FOXA2<sup>−</sup> cells compared with FOXA2<sup>+</sup> cells (Figures 3D, S3D, and S3E). To measure the degree of spatial correlation between FOXA2 expression versus WNT, FGF, or BMP signaling activities, we measured signal intensities along a line that bisected cells in NTOs, circling the lumen, then computed the Pearson correlation coefficients between FOXA2 and LEF1, pERK, or pSMAD1/5/9 signals, respectively (Figure 3E). We did not observe any type of correlation between FOXA2 and LEF1 or pERK signal. By contrast, a negative correlation was found between FOXA2 and pSMAD1/5/9 intensities, consistent with a role of BMP signaling in FOXA2 suppression. Taken together, the BMP pathway exhibited an appropriate activity pattern and perturbation phenotype to be further considered as a candidate signal by which intermediate FOXA2<sup>+</sup> clusters interact.

### BMP signaling regulates FOXA2 cluster resolution

To functionally investigate the role of BMP signaling during the phase of cluster competition, we perturbed the BMP pathway

from day 4 onward using pharmacological and genetic approaches (Figure 4A). To quantitatively assess the effect of perturbations on inter-cluster communication, we adapted our protocol to support the growth of larger NTOs (Figures S4A–S4C). These demonstrated a higher frequency of multiple rather than single FOXA2<sup>+</sup> clusters (Figure S4D) due to a positive correlation between NTO size and endpoint cluster number (Figure S4E).

BMP4 ligand treatment led to a dose-dependent decrease in the proportion of NTOs with any FOXA2<sup>+</sup> clusters persisting at day 6 (Figures 4B and 4C). Conversely, BMP pathway inhibition by application of recombinant NOGGIN or the small molecule LDN193189 (LDN) led to a mild increase in the total proportion of NTOs with any FOXA2<sup>+</sup> clusters (Figures 4B, 4C, and S3E). When broken down into whether each NTO contained 1, 2, or 3+ clusters, BMP inhibition resulted in a significant increase in the proportion of NTOs with 3+ endpoint clusters (Figure 4C). This is consistent with a role of endogenous BMP signaling in promoting FOXA2 cluster extinction, which is alleviated by BMP inhibition.

Given that larger and brighter clusters were more likely to persist (Figures 2G and 2H), we hypothesized that heterogeneous expression in BMP pathway genes as a function of FOXA2 expression levels may contribute to the competition. Using fluorescence-activated cell sorting (FACS), we sorted cells from NTOs according to their FOXA2-Venus levels into negative, low, medium, and high expression at day 4 and performed RNA-seq. Indeed, we observed an increase in expression of BMP ligands as well as inhibitors, corresponding to higher FOXA2 expression levels (Figure S4F). When analyzing expression in FACS-sorted FOXA2-Venus negative, low, medium, and high cells from intermediate (day 4) and clustered (day 5) NTOs, we found that by day 5 the tested BMP ligands, as well as inhibitors, showed positive correlation with FOXA2 expression levels or increased expression in FOXA2<sup>+</sup> compared with negative cells, which could explain why brighter FOXA2<sup>+</sup> clusters are more likely to “win” the competition (Figure S4G).

We then tested whether endogenously produced NOGGIN plays a role in regulating FOXA2<sup>+</sup> clusters in NTOs by generating *Noggin* mutant (*Nog<sup>mut</sup>*) ESCs with CRISPR-Cas9 (Figure S4H), then subjecting them to NTO formation and BMP pathway perturbations (Figures 4D–4F). A significantly lower proportion of *Nog<sup>mut</sup>* NTOs contained any endpoint FOXA2<sup>+</sup> clusters compared with WT NTOs (Figure 4E). BMP4 treatment led to a further and dose-dependent reduction in the proportion of *Nog<sup>mut</sup>* NTOs with any FOXA2<sup>+</sup> clusters (Figure 4E). Furthermore, when we quantified the size of FOXA2<sup>+</sup> clusters, *Nog<sup>mut</sup>* NTOs and BMP-treated WT NTOs both exhibited significantly smaller FOXA2<sup>+</sup> clusters than untreated WT NTOs (Figure 4F). Exogenous application of NOGGIN protein to *Nog<sup>mut</sup>* NTOs rescued the proportion of FOXA2<sup>+</sup> NTOs (Figure 4E) and FOXA2<sup>+</sup> cluster

pathway (GO: 0030509) based on Ensembl 94 annotation. Data point color indicates up-regulated in FOXA2<sup>+</sup> (green, #009988), up-regulated in FOXA2<sup>−</sup> (pink, #EE3377), or not significantly differentially regulated genes (light blue).

(D) Time course of NTOs fixed from days 3 to 5 (single z slice) showing a merged image at 10× magnification and a representative NTO (zoom) stained for DAPI (blue), FOXA2 (green), and LEF1 (left) or pERK (middle) or pSMAD1/5/9 (right) (magenta) to visualize WNT, FGF or BMP pathway activity respectively. Dashed white lines mark the outlines of the NTOs. Scale bars: 100 μm for 10× and 50 μm for zoom of representative NTOs.

(E) Analysis of Pearson correlation coefficient. Left: representative NTO used for the anti-correlation analysis performed on single z slices of images on day 5. For each NTO cross-section, a line with width of 10 pixels (shown in yellow) was drawn around the NTO, starting in the FOXA2<sup>+</sup> cluster and sparing the lumen. Scale bars, 100 μm. Right: intensity profiles of FOXA2 and LEF1/pERK/pSMAD were measured and analyzed by calculating the Pearson correlation coefficient by sampling the pixels for two channels (FOXA2 versus LEF1, FOXA2 versus pERK, FOXA2 versus pSMAD). NTOs are *n* = 56 LEF1, *n* = 49 pERK, *n* = 76 pSMAD. See also Figure S3.

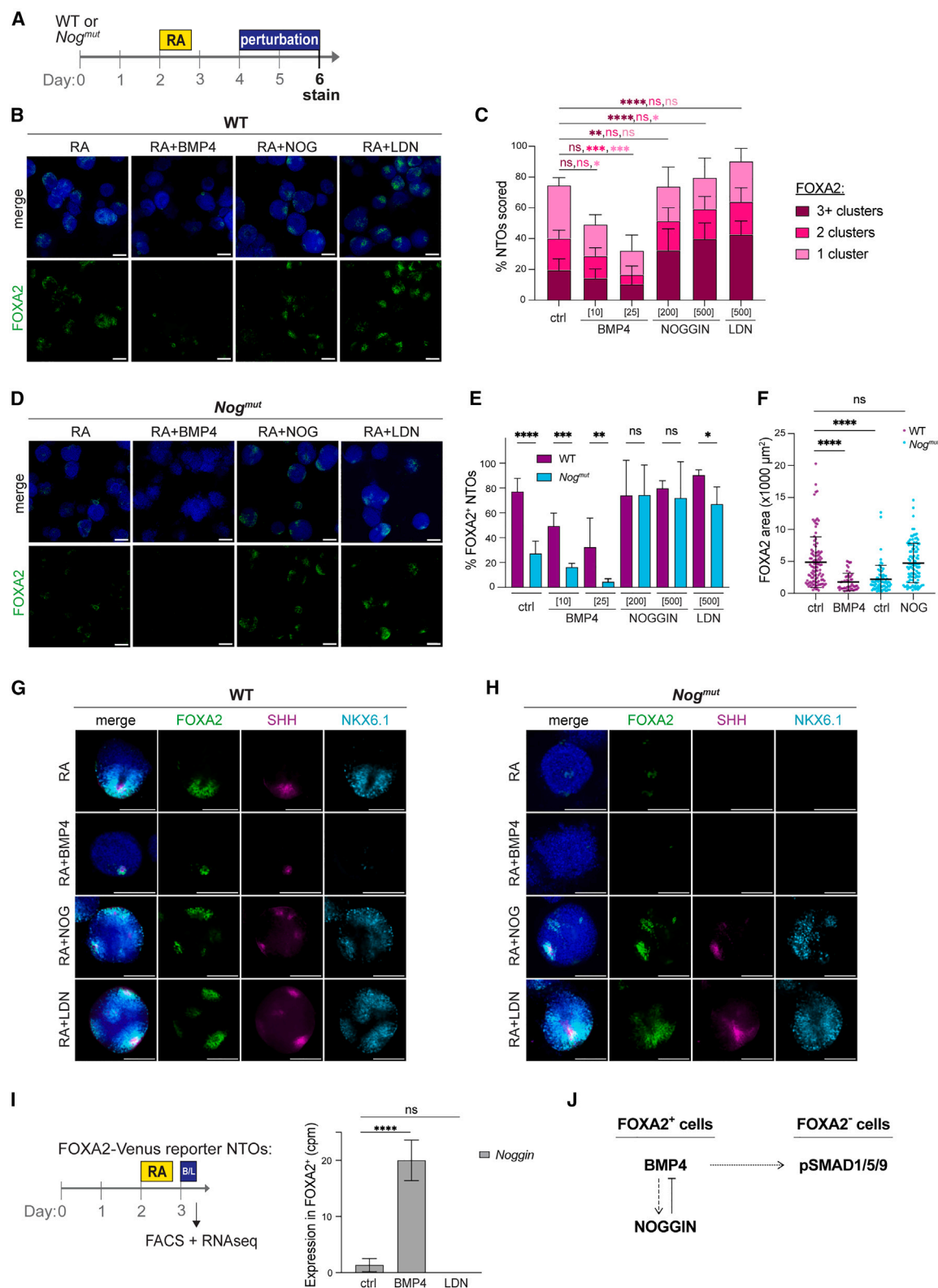


**Figure 4. BMP pathway activation or suppression modulates FOXA2 cluster persistence**

(A) Timeline of BMP pathway perturbations using “large NTO” protocol relevant for (B)–(H) (see Figure S4). Perturbations were performed from days 4 to 6 after an 18 h RA pulse from days 2 to 3.

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size (Figure 4F) to WT levels. We confirmed that the FOXA2 clusters in NOGGIN-treated *Nog<sup>mut</sup>* NTOs represent functional SHH-expressing floorplates at day 6 by co-staining for SHH and SHH/GLI-responsive NKX6.1 (Figures 4G and 4H).

Together, these data demonstrate that BMP signaling activity level regulates FOXA2<sup>+</sup> cluster outcomes during NTO self-organization. BMP pathway activation versus inhibition controlled FOXA2<sup>+</sup> cluster extinction versus persistence in a dose-dependent manner, as well as modulating FOXA2<sup>+</sup> cluster size. Endogenously, *Bmp4* ligand and BMP-inhibitor *Noggin* were both expressed by FOXA2<sup>+</sup> cells (Figure 3C), leading us to query the regulatory interactions between FOXA2, BMP4, and NOGGIN that underpin self-organization in NTOs. To examine how BMP signaling affects gene expression response in FOXA2<sup>+</sup> cells, we perturbed the BMP pathway in NTOs, then sorted according to FOXA2-Venus reporter level and performed RNA-seq after 8 h to capture early transcriptional responses in nascent FOXA2<sup>+</sup> cells (Figures 4I and S4I–S4K). FOXA2<sup>+</sup> cells from BMP4-treated NTOs showed a significant increase in *Noggin* expression (Figure 4I), in agreement with BMP-driven *Noggin* induction reported in human two-dimensional (2D) gastruloids,<sup>21</sup> indicating a feedback loop in which FOXA2<sup>+</sup> clusters protect themselves from the BMP ligands that they themselves produce (Figure 3C).

In summary, we propose a FOXA2-BMP4-NOGGIN signaling circuitry that underpins cluster competition during NTO self-organization (Figure 4J). FOXA2 cells express BMP ligands (Figure 3C) that, plausibly through pSMAD1/5/9, suppress FOXA2 in surrounding cells. NOGGIN, also expressed by FOXA2 cells (Figure 3C) and under feedback control by BMP (Figure 4J), promotes the persistence and size of winner clusters.

## NOGGIN regulates floorplate size in the anterior NT *in vivo*

Given our observations in NTOs, we asked whether deleting *Noggin* might reduce floorplate size *in vivo*. We crossed mice bearing *Noggin* floxed (*Nog<sup>Fl/Fl</sup>* or *Nog<sup>Fl/+</sup>*)<sup>22</sup> and *Sox2<sup>CreERT2/+</sup>*<sup>23</sup> alleles (Figures 5A and S5A), tamoxifen-treated at E5.5 to induce *Nog* conditional knockout (cKO) in the epiblast, and harvested embryos at E10.5 to evaluate FOXA2 and SHH expression in the NT at different anatomical levels (Figure 5B).

By quantifying the DAPI<sup>+</sup> area in cross-sections, we observed that mutant embryos exhibited slightly smaller NTs in the cervical spine region compared with control embryos from the same developmental stage (Figure S5B). To avoid any confounding effect, we measured the areas of FOXA2 and SHH protein expression (Figures S5C and S5D), then normalized to respective NT size (Figure 5C). We found a significant decrease in the normalized areas of FOXA2 and SHH expression in *Nog* cKO compared with control embryos, specifically in the mid/hindbrain region in which the floorplate forms prior to notochord development.<sup>24</sup> By contrast, there was no difference in the FOXA2 and SHH domain sizes in the notochord-dependent cervical and thoracic spinal cord. Altogether, our *in vitro* and *in vivo* findings suggest that floorplate establishment in the mid/hindbrain region is regulated via BMP-dependent cell communication.

## DISCUSSION

Here, we describe how a floorplate-signaling center self-organizes in clonal 3D NTOs. Following a pulse of RA signal, FOXA2<sup>+</sup> cells emerge in a salt-and-pepper fashion at day 3,

(B) Representative images at 10× magnification (single z slice) of WT NTOs stained for DAPI (blue) and FOXA2 (green) for RA, RA + BMP4 (25 ng/mL), RA + NOG (200 ng/mL), and RA + LDN (500 nM). Scale bars, 100 μm.

(C) 3D analysis of numbers of FOXA2<sup>+</sup> clusters in WT NTOs on day 6 shown in (B). Results were statistically analyzed using an ordinary one-way ANOVA and Dunnett's multiple comparisons test. For 3 or more clusters: RA versus RA + BMP4 (10 ng/mL) was n.s., RA versus RA + BMP4 (25 ng/mL) was n.s., RA versus RA + NOG (200 ng/mL) *p* = 0.0029, RA versus RA + NOG (500 ng/mL) *p* ≤ 0.0001, RA versus RA + LDN (500 nM) *p* ≤ 0.0001; for 2 clusters: RA versus RA + BMP4 (10 ng/mL) was n.s., RA versus RA + BMP4 (25 ng/mL) *p* = 0.0005, RA versus RA + NOG (200 ng/mL) was n.s., RA versus RA + NOG (500 ng/mL) was n.s., RA versus RA + LDN (500 nM) was n.s.; for 1 cluster: RA versus RA + BMP4 (10 ng/mL) *p* = 0.0171, RA versus RA + BMP4 (25 ng/mL) *p* = 0.0007, RA versus RA + NOG (200 ng/mL) was n.s., RA versus RA + NOG (500 ng/mL) *p* = 0.0153, RA versus RA + LDN (500 nM) was n.s. Concentration [x] expressed in ng/mL for BMP4 and NOGGIN, or in nM for LDN. Asterisks indicate statistical significance, n.s. indicates non-significance.

(D) Representative images at 10× magnification (single z slice) of *Nog<sup>mut</sup>* NTOs stained for DAPI (blue) and FOXA2 (green) upon RA, RA + BMP4 (25 ng/mL), RA + NOG (200 ng/mL), and RA + LDN (500 nM). Scale bars, 100 μm.

(E) 3D analysis of the proportion of FOXA2<sup>+</sup> NTOs shown in (B) and (D) of WT (purple) and *Nog<sup>mut</sup>* (cyan). Results were statistically analyzed using an ordinary one-way ANOVA, always comparing WT with *Nog<sup>mut</sup>* samples. RA versus RA *p* ≤ 0.0001, RA + BMP4 (10 ng/mL) versus RA + BMP4 (10 ng/mL) *p* = 0.0004, RA + BMP4 (25 ng/mL) versus RA + BMP4 (25 ng/mL) *p* = 0.0039, RA + LDN (500 nM) versus RA + LDN (500 nM) *p* = 0.0229, while the other conditions were n.s. A total of *n* = 3,407 (between 306 and 414 per condition) WT NTOs and *n* = 3,425 (between 328 and 415 per condition) *Nog<sup>mut</sup>* NTOs were included in the analysis. Concentration [x] expressed in ng/mL for BMP4 and NOGGIN, or in nM for LDN. Asterisks indicate statistical significance, n.s. indicates non-significance.

(F) 3D analysis of average surface area of FOXA2<sup>+</sup> clusters in WT (purple) and *Nog<sup>mut</sup>* (cyan) NTOs. Each dot represents an average cluster area in a given NTO. Results were statistically analyzed using Kruskal-Wallis test and Dunn's multiple comparisons test comparing RA with RA + BMP4 (in WT NTOs), RA and RA + NOG (in *Nog<sup>mut</sup>* NTOs). WT RA versus WT RA + BMP4 *p* ≤ 0.0001, WT RA versus *Nog<sup>mut</sup>* RA *p* ≤ 0.0001, and WT RA versus *Nog<sup>mut</sup>* RA + NOG was n.s. Asterisks indicate statistical significance, n.s. indicates non-significance.

(G) Representative images (single z slice) of a day 6 WT NTO stained for DAPI (blue), FOXA2 (green), SHH (magenta), and NKX6.1 (cyan) for RA, RA + BMP4 (25 ng/mL), RA + NOG (200 ng/mL), and RA + LDN (500 nM). Scale bars, 100 μm.

(H) Representative images (single z slice) of a day 6 *Nog<sup>mut</sup>* NTO stained for DAPI (blue), FOXA2 (green), SHH (magenta), and NKX6.1 (cyan) for RA, RA + BMP4 (25 ng/mL), RA + NOG (200 ng/mL), and RA + LDN (500 nM). Scale bars, 100 μm.

(I) Transcriptional response in FOXA2<sup>+</sup> cells after BMP signal activation or suppression shows FOXA2<sup>+</sup> cells induce *Noggin* expression after BMP exposure. Schematic: at day 3, BMP perturbations were added for 8 h before NTOs were dissociated and FACS sorted into FOXA2<sup>+</sup> and FOXA2<sup>−</sup> cells for RNA analysis. Expression levels (cpm) of *Noggin* in FOXA2<sup>+</sup> cells upon RA, RA + BMP4 (1.5 ng/mL), and RA + LDN (100 nM) treatments. Results were statistically analyzed using DESeq2 with pairwise comparison, RA + BMP4 versus RA *p* ≤ 0.0001 for *Noggin* expression. Asterisks indicate statistical significance, n.s. indicates non-significance.

(J) Model of conceptual interactions between BMP4 and NOGGIN activities in FOXA2<sup>+</sup> cells and their interaction via pSMAD1/5/9 activity in FOXA2<sup>−</sup> cells. See also Figure S4.

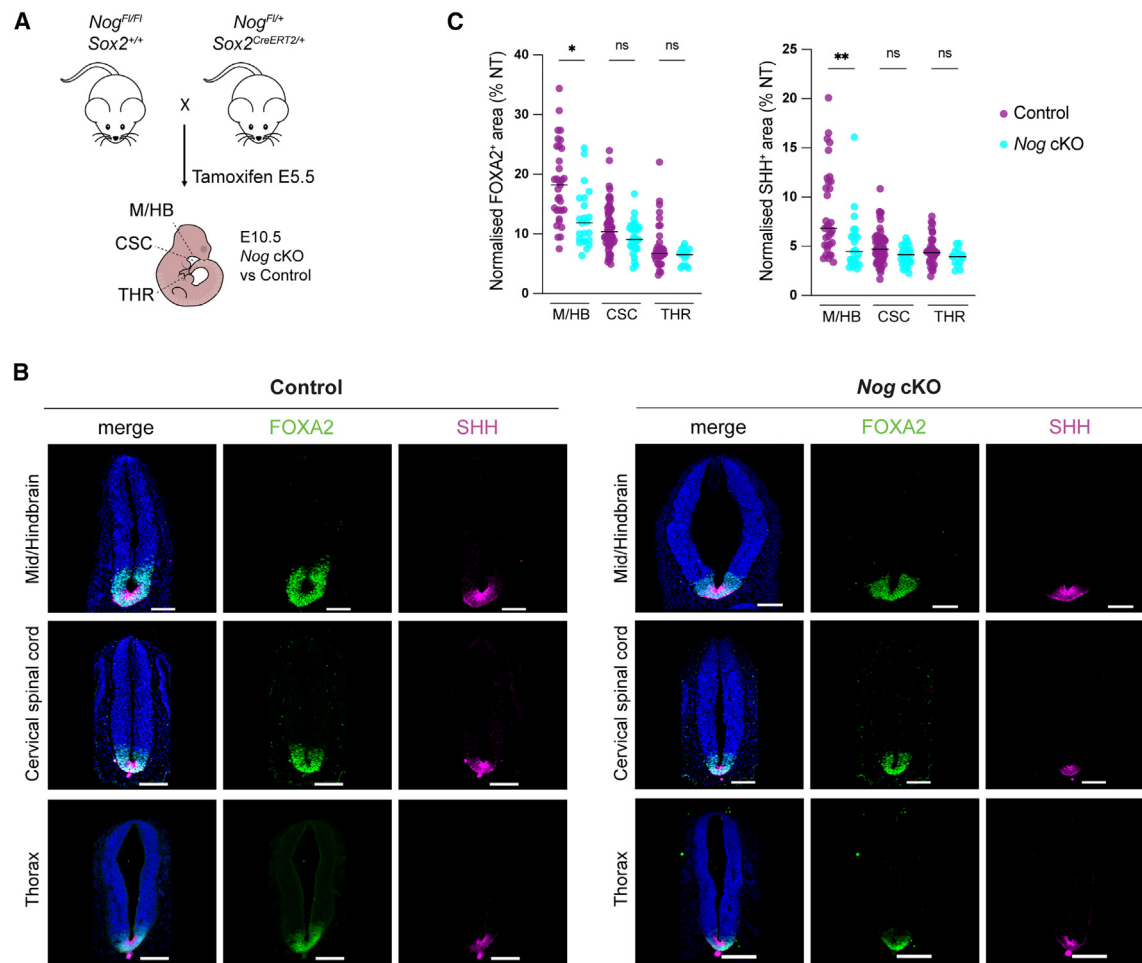


**Figure 5. In vivo role of NOGGIN in anterior floorplate size in the mouse embryo**

(A) Schematic of mouse crossing performed for inducible deletion of *Noggin*. E10.5 mouse showing the anatomy and section planes of the harvested and analyzed embryos.

(B) Representative images of E10.5 control (*Nog<sup>Fl/Fl</sup>; Sox2<sup>+/+</sup>* or *Nog<sup>Fl/Fl</sup>; Sox2<sup>CreERT2/+</sup>*) and *Nog cKO* (*Nog<sup>Fl/Fl</sup>; Sox2<sup>CreERT2/+</sup>*) embryos of mid/hindbrain (M/HB), cervical spinal cord (CSC), and thorax (THR) regions stained for DAPI (blue), FOXA2 (green), and SHH (magenta). Scale bars, 100  $\mu$ m.

(C) Normalized area of FOXA2 and SHH at different anterior-posterior levels of the embryonic NT. Quantification was made on serial sections of 6 control embryos (136 sections total, 33 for M/HB, 62 for CSC, 33 for THR) in purple and 3 *Nog cKO* embryos (68 sections total, 23 for M/HB, 30 for CSC, 15 for THR) in cyan from two litters. Kruskal-Wallis test and Dunn's multiple comparison tests were performed between anatomical regions of control versus *Nog cKO* (FOXA2: M/HB versus M/HB  $p = 0.0165$ , CSC versus CSC  $p = 0.2706$ , THR versus THR  $p = 0.3926$ , SHH: M/HB versus M/HB  $p = 0.0031$ , CSC versus CSC  $p = 0.0792$ , THR versus THR  $p = 0.4484$ ). Horizontal bars represent means of the data, asterisks indicate statistical significance, n.s. indicates non-significance.

then undergo a process of cluster formation and resolution to form a localized domain in which SHH morphogen expression commences from day 5 (Figures 1 and 2). During NTO self-organization, intermediate FOXA2<sup>+</sup> clusters do not behave independently but instead promote each other's extinction (Figure 2) via a BMP-mediated signaling mechanism, while NOGGIN protects FOXA2 (Figures 3 and 4). Furthermore, BMP4 application or *Noggin* mutation decreases FOXA2 domain size, which can be rescued by exogenous NOGGIN (Figure 4). Strikingly, the expression of both BMP ligands and inhibitors is enriched in FOXA2<sup>+</sup> cells, whereas BMP signal transduction indicated by pSMAD1/5/9 is enriched in FOXA2<sup>-</sup> cells (Figure 3). This represents a self-organization mechanism in which spatially scattered FOXA2<sup>+</sup> cells express the signaling machinery driving their competition to result in a ventral pole.

We imaged this dynamic process and found that cells follow two major processes, cluster competition and cell sorting, which might be linked to a considerable heterogeneity in cluster size and FOXA2 expression level. Given that we identified the BMP4-NOGGIN circuit as a key player in cluster competition, we examined expression of these factors and saw correspondence with FOXA2 expression intensity. Correspondence between *Noggin* and FOXA2 first appeared in day 5 NTOs, while on day 4, *Noggin* expression was higher in FOXA2 low cells. It will be interesting to further understand whether this small, statistically significant difference in FOXA2 low cells on day 4 contributes to the dynamics of self-organization and whether heterogeneity in cluster size and brightness could relate to differential BMP circuit expression. Future work will be aimed at understanding cluster competition in NTOs more deeply, such as

how FOXA2 levels or cluster size are controlled, how the BMP-NOGGIN circuit interfaces with the sorting and differential cadherin expression, and whether one of these two processes might dominate over the other in different phases of the self-organization. Given the known tendency of cells to sort based on differential cadherin expression,<sup>25</sup> this observation may provide an explanation for the cell sorting observed in the NTOs. However, other recent advances have been reported to control floorplate formation in the mouse *in vivo* by integrating mechanical cues and translating these into cell-specific transcriptional programs, which could potentially also play a role in cell sorting of FOXA2 in the NTOs.<sup>26</sup>

Expression of both the FOXA2 “inhibitor” (BMP4) and “activator”/protector (NOGGIN) molecules from the same cells (FOXA2<sup>+</sup>), and the feedback by which BMP4 promotes *Noggin* expression in FOXA2<sup>+</sup> cells (Figure 4), displays features of a reaction-diffusion (RD) mechanism.<sup>27</sup> In the future, it will be interesting to test the extent to which an RD-type mechanism underpins floorplate patterning in NTOs. A thorough understanding will involve developing the means to determine the localization and dynamics of BMP pathway components. This NTO system offers the intriguing possibility to define, quantitatively, how the relative contributions of long-range communication versus physical cell sorting mechanisms (Figure 2) complement each other to promote self-organizational robustness. How our findings from mouse ESCs compare with recently described human NTOs that induce full or partial dorsoventral patterning will also be important.<sup>28–30</sup> In these studies, fixed time point analysis showed scattered FOXA2 prior to floorplate formation, but as less than 30% of human NTOs formed floorplates, it has been unclear whether the scattered starting point was an intermediate to the final state or whether distinct subpopulations arose over time.<sup>28</sup> WNT signaling has been proposed to act in an RD patterning system in human NTOs<sup>30</sup>—something that we did not observe in mouse NTOs. Whether these discrepancies are due to experimental approaches or whether they highlight intriguing inter-species differences remains to be addressed.

Other 3D organoid models that form from aggregates of 100s–1,000s of cells<sup>31–35</sup> can self-organize by amplifying initial asymmetries, yielding persistence of a signaling center at the location of its initial emergence. In contrast, our NTOs form clonally from single ESCs and show a regulative self-organization process, with nascent clusters competing via long-range signaling prior to establishing a stable ventral organizer. To our knowledge, cluster competition has not been observed in other organoid systems, although few studies have yet investigated the earliest stages of their self-organization due to constraints in efficiency, topographic predictability, or optical density that have limited tractability. Recent work addressing how 3D gastruloids initiate self-organization of an anterior-posterior axis<sup>36,37</sup> have combined improvements in homogeneity of input cells and analytical tools to elucidate how early variation in Nodal/BMP and cell position act prior to heterogeneity in WNT activity, which determines which cells will form the posterior pole, reinforced by cell sorting. Endogenous and exogenous BMP signaling have been shown to mediate organized lineage induction in 3D models of human epiblast<sup>38,39</sup> and mouse appendages,<sup>40</sup> but again without evidence of cluster competition. The processes we describe here are also distinct from those found in clonal in-

testinal organoids, where local activation of YAP1 induces symmetry breaking via DLL1 to determine the future crypt<sup>41</sup> without indication of competition between emergent DLL1 sites. An interesting question is whether mechanisms such as those we describe may be upstream of self-organization mechanisms in other organoids.

Here, we have identified and tested the importance of BMP signaling for cluster interactions in mouse NTOs. Because BMP is a key morphogen driving dorsal patterning of the NT from the dorsal roofplate organizer,<sup>42–44</sup> it seems surprising to observe BMP ligand expression in ventral floorplate cells. However, BMP ligands are reported to be expressed in the *in vivo* floorplate,<sup>45–47</sup> in keeping with the enrichment of BMP ligand expression we observe in FOXA2<sup>+</sup> cells of NTOs (Figure 3). Our observation that BMP4 and NOGGIN regulate floorplate formation and size in NTOs led us to investigate whether BMP signaling circuitry plays a role in floorplate formation *in vivo*. cKO of *Noggin* in the epiblast led to a reduction in floorplate size at E10.5, particularly at mid/hindbrain regions of the NT where the floorplate, like our NTOs, forms in the absence of the underlying notochord (Figure 5). This raises the possibility that ventral sources of BMP may also contribute to NT patterning defects in mouse mutants of the BMP pathway.<sup>45–47</sup>

The broader applicability of the BMP-NOGGIN circuit regulating floorplate formation in NTOs highlights the potential of tractable *in vitro* self-organizing systems to uncover mechanisms that may contribute *in vivo*, by isolating or exaggerating such mechanisms away from the complexity of the developing embryo. This raises fascinating possibilities for the future: by integrating classical developmental biology with self-organization in stem cell models, we can quantitatively tease apart how embryonic context and inductive cues interface with self-organizing modules to allow regulative yet robust outcomes of vertebrate development.

### Limitations of the study

In this study, we suggest differential cadherin expression as a possible explanation for the physical cell sorting of FOXA2<sup>+</sup> cells occurring during self-organization. However, this might not be the only mechanism, and other (mechanical) cues could potentially contribute to cell sorting. When considering features that predict which cluster will win or lose during competition, we investigated cluster size and FOXA2 expression, but other parameters could also be involved. Additionally, we could not define the exact spatial localization of BMP4 and NOGGIN proteins due to the lack of functioning specific antibodies. Finally, the role of FOXA2<sup>−</sup> cells in the self-organization process is still unknown.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.devcel.2024.04.021>.

## ACKNOWLEDGMENTS

We thank P. Pasierbek, A.C. Moreno, T. Lendl, and K. Aumayr for microscopy support; G. Schmauss for FACS support; M. Novatchkova for assistance with Bioinformatic analyses; J. Ahel, A. Polikarpova, S. Horer, E. Cesare, and E. Norouzi for technical assistance; A. Meinhardt for supervision; DRESDEN-concept Genome Center, A. Vogt, A. Sommer, and the Vienna BioCenter NGS facility for RNA sequencing. We are grateful to M. Placzek and E. Martí for discussions about the floorplate; to S. Shvartsman for valuable input; to A. Aszodi, W. Masselink, and S. Raiders for advice on statistical analyses; to J. Cornwall Scoones, G. Martello, and Tanaka lab members for critical reading of the manuscript; E. Bassat and E. Chatzidaki for contributing schematics; and to K. Lust for support. This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement ERC AdG 742046) to E.M.T. This research was funded in whole or in part by the Austrian Science Fund (FWF) (10.55776/F7803-B) (Stem Cell Modulation) to E.M.T. and A.K., Sir Henry Wellcome postdoctoral fellowship to H.T.S., ELBE fellowship to K.I., and National Science Foundation (US) Phy 2013131 to E.S. The A.K. lab is also supported by ISTA and the European Research Council under Horizon Europe grant 101044579, and S.L. is supported by Gesellschaft für Forschungsförderung Niederösterreich m.b.H. fellowship SC19-011. This work was supported in part by the Francis Crick Institute, which receives its core funding from Cancer Research UK (CC001051), the UK Medical Research Council (CC001051), and the Wellcome Trust (CC001051). For the purpose of open access, the authors have applied a CC BY public copyright license to any author accepted manuscript (AAM) version arising from this submission.

## AUTHOR CONTRIBUTIONS

T.K., H.T.S., and E.G. designed and performed experiments, created methodologies, and analyzed the data. K.I. provided supervision, set up live imaging conditions, developed image analysis pipelines, and helped to analyze data. D.C. and E.S. developed image analysis pipelines. M.M. and J.B. generated *Shh*<sup>-/-</sup> ESCs. F.B.P. performed and bioinformatically analyzed BRB sequencing. J.W. performed bioinformatic analyses. E.C. and L.A. performed experiments. S.L. and A.K. provided *Nog* cKO embryos. A.H. and R.A.N. assisted with the pharmacological screen. N.E. supported the development of larger NTO media. T.K., H.T.S., and E.M.T. wrote the manuscript. E.M.T. conceived and supervised the study.

## DECLARATION OF INTERESTS

A.H. and R.A.N. are full-time employees of Boehringer Ingelheim RCV.

Received: August 25, 2023

Revised: March 8, 2024

Accepted: April 30, 2024

Published: May 21, 2024

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                                                                       | SOURCE                       | IDENTIFIER                   |
|-------------------------------------------------------------------------------------------|------------------------------|------------------------------|
| <b>Antibodies</b>                                                                         |                              |                              |
| Monoclonal rabbit anti-FOXA2                                                              | Cell Signalling Technologies | Cat# 8186; RRID:AB_10891055  |
| Polyclonal goat anti-FOXA2                                                                | R&D                          | Cat# AF2400; RRID:AB_2294104 |
| Monoclonal rabbit anti-SHH                                                                | Cell Signalling Technologies | Cat# 2207; RRID:AB_2188191   |
| Monoclonal mouse anti-NKX6.1                                                              | DSHB                         | Cat# F55A10; RRID:AB_532378  |
| Monoclonal rabbit anti-LEF1                                                               | Cell Signalling Technologies | Cat# 2230; RRID:AB_823558    |
| Monoclonal rabbit anti-Phospho-SMAD1 (Ser463/465)/ SMAD5 (Ser463/465)/ SMAD9 (Ser465/467) | Cell Signalling Technology   | Cat# 13820; RRID:AB_2493181  |
| Monoclonal rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)                       | Cell Signalling Technology   | Cat# 4370; RRID:AB_2315112   |
| Monoclonal rabbit anti-E-Cadherin                                                         | Cell Signalling Technology   | Cat# 3195; RRID:AB_2291471   |
| Monoclonal rat anti-N-Cadherin                                                            | DSHB                         | Cat# MNCD2; RRID:AB_528119   |
| Monoclonal mouse anti-β-Tubulin                                                           | MPI-CBG                      | N/A                          |
| Alexa Fluor conjugated secondary antibodies for IHC (Donkey anti-Mouse/Goat/Rabbit)       | Invitrogen                   | N/A                          |
| HRP conjugated secondary antibodies for WB (Goat anti-Rabbit/Mouse)                       | Invitrogen                   | N/A                          |
| DAPI                                                                                      | Sigma-Aldrich                | Cat# D9542                   |
| <b>Chemicals, peptides, and recombinant proteins</b>                                      |                              |                              |
| N-2 Supplement (100X)                                                                     | Gibco                        | Cat# 17502048                |
| B-27™ Supplement (50X), serum free                                                        | Gibco                        | Cat# 17504044                |
| DMEM/F-12, no glutamine                                                                   | Gibco                        | Cat# 21331020                |
| Neurobasal™ Medium                                                                        | Gibco                        | Cat# 21103049                |
| L-Glutamine (200 mM)                                                                      | Gibco                        | Cat# A2916801                |
| 2-Mercaptoethanol (50 mM)                                                                 | Gibco                        | Cat# 31350010                |
| Penicillin-Streptomycin Solution                                                          | Gibco                        | Cat# 15140130                |
| CHIR 99021                                                                                | Tocris                       | Cat# 4423                    |
| PD 0325901                                                                                | Tocris                       | Cat# 4192                    |
| Mouse LIF                                                                                 | Qkine                        | Cat# Qk018-1000              |
| Accutase                                                                                  | Invitrogen                   | Cat# 00-4555-56              |
| Sodium Pyruvate (100 mM)                                                                  | Gibco                        | Cat# 11360070                |
| BMP4                                                                                      | R&D                          | Cat# PHC9534                 |
| BMP7                                                                                      | R&D                          | Cat# 354-GMP                 |
| LDN-193189                                                                                | Selleck Chemicals            | Cat# S2618                   |
| Noggin                                                                                    | R&D                          | Cat# 6057-NG-025             |
| FGF2                                                                                      | PeproTech                    | Cat# 100-18B                 |
| FGF8b                                                                                     | In house                     | N/A                          |
| IWP-2                                                                                     | Selleck Chemicals            | Cat# S7085                   |
| IWR-1endo                                                                                 | Merck                        | Cat# I0161                   |
| Gelatin                                                                                   | Merck                        | Cat# G2500                   |
| Puromycin -dihydrochlorid                                                                 | Thermo Scientific            | Cat# P9620                   |
| Neomycin                                                                                  | Merck                        | Cat# N1142                   |
| Geneticin (G418)                                                                          | Invivogen                    | Cat# ant-gn-1                |
| Trypsin-EDTA (0.25%)                                                                      | Gibco                        | Cat# 25200056                |
| Albumin (BSA) Fraction V (pH 7.0)                                                         | Panreac AppliChem            | Cat# A1391                   |

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**Continued**

| REAGENT or RESOURCE                                              | SOURCE                         | IDENTIFIER         |
|------------------------------------------------------------------|--------------------------------|--------------------|
| Normal Donkey Serum                                              | Jackson ImmunoResearch         | Cat# 017-000-121   |
| Target Retrieval Solution, Citrate pH 6                          | Dako                           | Cat# S2369         |
| Advanced DMEM/F-12                                               | Gibco                          | Cat# 12634010      |
| MEM Non-Essential Amino Acids Solution (100X)                    | Gibco                          | Cat# 11140035      |
| Basement Membrane Matrix (Matrigel)                              | Corning                        | Cat# BDL354234     |
| CellCarrier-96 Ultra Microplates                                 | PerkinElmer                    | Cat# 6055302       |
| 35 mm Dish   No. 1.5 Coverslip   14 mm Glass Diameter   Uncoated | MatTek Corporation             | Cat# P35G-1.5-14-C |
| All-Trans Retinoic Acid                                          | Merck                          | Cat# R2625         |
| Paraformaldehyde, reagent grade, crystalline                     | Merck                          | Cat# P6148         |
| EMS 8% Paraformaldehyde Aqueous Solution, EM Grade               | Electron Microscopy Sciences   | Cat# 157-8         |
| CUBIC-R+(N)                                                      | Matsumoto et al. <sup>48</sup> | N/A                |
| Propyl gallate, for microscopy, ≥98.0% (HPLC)                    | Merck                          | Cat# 02370         |
| Prolong™ Diamond Antifade Mountant                               | Invitrogen                     | Cat# P36965        |
| Draq7™                                                           | Biostatus                      | Cat# DR71000       |
| Rock inhibitor StemMACS Y27632                                   | Miltenyi Biotec                | Cat# 130-103-922   |
| Tamoxifen                                                        | Sigma-Aldrich                  | Cat# T5648         |
| TrypLE Select Enzyme (1X), no phenol red                         | Gibco                          | Cat# 50-591-419    |
| NuPAGE 4-12% Bis-Tris gel                                        | Invitrogen                     | Cat# NP0322BOX     |

**Critical commercial assays**

|                                                     |                           |                   |
|-----------------------------------------------------|---------------------------|-------------------|
| RNeasy Mini Kit                                     | Qiagen                    | Cat# 74104        |
| DNase I                                             | Qiagen                    | Cat# 79254        |
| SuperScript VILO™ cDNA Synthesis Kit                | Invitrogen                | Cat# 11754050     |
| Poly(A) mRNA Magnetic Isolation Module              | New England Biolabs (NEB) | Cat# E7490        |
| Ultra Directional RNA Library Prep Kit for Illumina | New England Biolabs (NEB) | Cat# E7420        |
| HS RNA Kit                                          | Agilent                   | Cat# DNF-472-0500 |
| KAPA HiFi HS RM (100 x 25 rxns)                     | Roche                     | Cat# KK2601       |
| Paramagnetic beads-based DNA purification Kit       | In house                  | N/A               |
| Recombinant RNasin(R) RNase Inhibitor, 2,500u       | Promega                   | Cat# N2511        |
| Maxima H Minus Reverse Transcriptase (200 U/L)      | Thermo Scientific         | Cat# EP0751       |
| Exonuclease I (E.coli)                              | New England Biolabs (NEB) | Cat# M0293S       |
| P3 Primary Cell 4D X Kit L (12 RCT)                 | Lonza                     | Cat# V4XP-3012    |

**Deposited data**

|                                                   |                               |                |
|---------------------------------------------------|-------------------------------|----------------|
| RNA-Seq data related to <a href="#">Figure 3</a>  | This study                    | GEO: GSE221187 |
| RNA-Seq data related to <a href="#">Figure 4</a>  | This study                    | GEO: GSE236543 |
| RNA-Seq data related to <a href="#">Figure S5</a> | This study                    | GEO: GSE260579 |
| RNA-Seq data related to <a href="#">Figure S1</a> | Ishihara et al. <sup>19</sup> | GEO: GSE214368 |

**Experimental models: Cell lines**

|                                            |                                 |                |
|--------------------------------------------|---------------------------------|----------------|
| Mus musculus (Male): R1                    | Nagy et al. <sup>49</sup>       | Cat# SCRC-1011 |
| Mus musculus (Male): HM1                   | Doetschman et al. <sup>50</sup> | N/A            |
| FOXA2-Venus, H2A-mCherry (R1 background)   | This study                      | N/A            |
| <i>Shh</i> <sup>-/-</sup> (HM1 background) | This study                      | N/A            |
| <i>Nog</i> <sup>mut</sup> (R1 background)  | This study                      | N/A            |

**Experimental models: Organisms/strains**

|                                            |                               |                                       |
|--------------------------------------------|-------------------------------|---------------------------------------|
| <i>Nog</i> <sup>tm1.1Rmh/J</sup>           | Stafford et al. <sup>22</sup> | Strain #:016117, RRID:IMSR_JAX:016117 |
| <i>Sox2</i> <sup>tm1(cre/ERT2)Hoch/J</sup> | Arnold et al. <sup>23</sup>   | Strain #:017593, RRID:IMSR_JAX:017593 |

**Oligonucleotides**

|                                                        |               |     |
|--------------------------------------------------------|---------------|-----|
| gRNAs used in this study, see <a href="#">Table S1</a> | Sigma-Aldrich | N/A |
|--------------------------------------------------------|---------------|-----|

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### Continued

| REAGENT or RESOURCE                                   | SOURCE                              | IDENTIFIER                                                                                                                                                                                                            |
|-------------------------------------------------------|-------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| qPCR primers used in this study, see Table S1         | Sigma-Aldrich                       | N/A                                                                                                                                                                                                                   |
| Genotyping primers used in this study, see Table S1   | Sigma-Aldrich                       | N/A                                                                                                                                                                                                                   |
| Primers for cloning of FoxA2-Venus line, see Table S1 | Sigma-Aldrich                       | N/A                                                                                                                                                                                                                   |
| <b>Recombinant DNA</b>                                |                                     |                                                                                                                                                                                                                       |
| FoxA2-Venus Fusion (FVF) E-85 plasmid                 | Burtscher et al. <sup>51</sup>      | N/A                                                                                                                                                                                                                   |
| pSpCas9(BB)-2A-Puro (PX459)                           | Ran et al. <sup>52</sup>            | Addgene plasmid #48139; RRID:Addgene_48139                                                                                                                                                                            |
| pR26 CAG AsiSI/MluI                                   | Chu et al. <sup>53</sup>            | Addgene plasmid #74286; RRID:Addgene_74286                                                                                                                                                                            |
| pR26-sA-DEST                                          | This study                          | N/A                                                                                                                                                                                                                   |
| pR26-sA-H2-mCherry                                    | This study                          | N/A                                                                                                                                                                                                                   |
| pSpCas9(BB)-2A-Puro (PX459) V2.0                      | Ran et al. <sup>52</sup>            | Addgene plasmid #62988; RRID:Addgene_62988                                                                                                                                                                            |
| U6-IT-EF1As-Cas9-P2A-GFP                              | This study                          | N/A                                                                                                                                                                                                                   |
| <b>Software and algorithms</b>                        |                                     |                                                                                                                                                                                                                       |
| Fiji/ ImageJ (v2.9.0)                                 | Schindelin et al. <sup>54</sup>     | <a href="https://imagej.net/software/fiji/">https://imagej.net/software/fiji/</a>                                                                                                                                     |
| Jupyter lab (v3.4.8)                                  | Jupyter                             | <a href="https://jupyter.org/">https://jupyter.org/</a>                                                                                                                                                               |
| Python (v3.9.12)                                      | Python                              | <a href="https://python.org/">https://python.org/</a>                                                                                                                                                                 |
| R studio                                              | R Core Team                         | <a href="https://www.R-project.org/">https://www.R-project.org/</a>                                                                                                                                                   |
| CellProfiler 4                                        | Stirling et al. <sup>55</sup>       | N/A                                                                                                                                                                                                                   |
| MATLAB R2021a                                         | The MathWorks                       | N/A                                                                                                                                                                                                                   |
| GraphPad prism 9                                      | Dotmatics                           | N/A                                                                                                                                                                                                                   |
| CRISPick                                              | Broad Institute                     | <a href="https://portals.broadinstitute.org/gppx/crispick/public">https://portals.broadinstitute.org/gppx/crispick/public</a>                                                                                         |
| VBC score                                             | Michlits et al. <sup>56</sup>       | <a href="https://www.vbc-score.org/">https://www.vbc-score.org/</a>                                                                                                                                                   |
| Bowtie 2 (v2.3.4.1)                                   | Langmead and Salzberg <sup>57</sup> | <a href="https://bowtie-bio.sourceforge.net/bowtie2/index.shtml">https://bowtie-bio.sourceforge.net/bowtie2/index.shtml</a>                                                                                           |
| STAR (v2.6.0c, v2.5.2a, v2.7.9a)                      | N/A                                 | <a href="https://github.com/alexdobin/STAR">https://github.com/alexdobin/STAR</a>                                                                                                                                     |
| featureCounts subread (v1.6.2)                        | N/A                                 | <a href="https://subread.sourceforge.net/">https://subread.sourceforge.net/</a>                                                                                                                                       |
| DESeq2 (v1.18.1, v1.26.0, v1.42.0)                    | Love et al. <sup>58</sup>           | <a href="https://bioconductor.org/packages/release/bioc/html/DESeq2.html">https://bioconductor.org/packages/release/bioc/html/DESeq2.html</a>                                                                         |
| Trim Galore (v0.5.0)                                  | N/A                                 | <a href="https://github.com/FelixKrueger/TrimGalore">https://github.com/FelixKrueger/TrimGalore</a>                                                                                                                   |
| GRCm38 genome (Ensembl release 94)                    | N/A                                 | <a href="https://www.ensembl.org/Mus_musculus/Info/Index">https://www.ensembl.org/Mus_musculus/Info/Index</a>                                                                                                         |
| Clusterprofiler 4.6.2                                 | Yu et al. <sup>59</sup>             | <a href="https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html">https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html</a>                                                       |
| UMI-tools (v1.0.0)                                    | N/A                                 | <a href="https://github.com/CGATOxford/UMI-tools">https://github.com/CGATOxford/UMI-tools</a>                                                                                                                         |
| HTSeq (v0.11.2)                                       | Anders et al. <sup>60</sup>         | <a href="http://www-huber.embl.de/HTSeq">http://www-huber.embl.de/HTSeq</a>                                                                                                                                           |
| bcl2fastq                                             | N/A                                 | <a href="https://emea.support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html">https://emea.support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html</a> |
| SeqKit (v2.7.0)                                       | Shen et al. <sup>61</sup>           | <a href="https://bioinf.shenwei.me/seqkit/usage/">https://bioinf.shenwei.me/seqkit/usage/</a>                                                                                                                         |
| Cutadapt (v1.18)                                      | Martin <sup>62</sup>                | <a href="https://cutadapt.readthedocs.io/en/stable/">https://cutadapt.readthedocs.io/en/stable/</a>                                                                                                                   |
| GO.db 3.16.0                                          | N/A                                 | <a href="https://bioconductor.org/packages/release/data/annotation/html/GO.db.html">https://bioconductor.org/packages/release/data/annotation/html/GO.db.html</a>                                                     |

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Elly Tanaka ([elly.tanaka@imba.oeaw.ac.at](mailto:elly.tanaka@imba.oeaw.ac.at)).

#### Materials availability

Reagents such as cell lines and plasmids generated in this study are available; requests for materials should be addressed to the [lead contact](#).

### Data and code availability

RNA-seq data generated in this study have been deposited at GEO under accession codes GSE221187 (FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells at Day 5, [Figure 3](#)), GSE236543 (FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells after BMP perturbations, [Figure 4](#)), and GSE260579 (RNA-seq of FOXA2<sup>-</sup> versus FOXA2<sup>+</sup> low, medium, high cells, [Figure S4](#)) and RNA-seq analyses are provided in [Table S2](#).

This paper does not report original code.

Any additional information required to re-analyse the data reported in this paper is available from the [lead contact](#) upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Cell culture

#### Cell lines

R1 mouse embryonic stem cell (ESC) line<sup>49</sup> was used for most experiments and as the parental line for FOXA2-Venus reporter and FOXA2-Venus and H2A-mCherry double-reporter generation, and for *Noggin* mutant (*Nog*<sup>mut</sup>). HM1 ESC background<sup>50</sup> was used as the parental line for *Shh* knock-out (*Shh*<sup>-/-</sup>). Cell lines were routinely tested and confirmed negative for mycoplasma.

#### Maintenance

ESCs were cultured in N2B27+2i+LIF (2iLIF) and incubated at 37°C in 5% CO<sub>2</sub>. N2B27 medium comprised 1:1 DMEM/F-12 and Neurobasal (Gibco), 0.5% v/v N2 (Gibco), 1% v/v B27 (Gibco), 2 mM L-glutamine (Gibco), 1% penicillin/ streptomycin (Sigma), and 0.1 mM β-mercaptoethanol. To obtain 2iLIF medium, N2B27 was supplemented with 3 μM CHIR99021 (Tocris), 1 μM PD0325901 (Tocris) and 20 ng/ml murine LIF (Qkine). Tissue-culture treated 6-well plates were pre-coated with 0.15% gelatin (Sigma) in PBS at room temperature. Mouse ESCs were seeded at a density of 30,000 cells per 6-well, media was exchanged daily, and cells were passaged every third day by incubation with Accutase (Gibco) for 3 mins at 37°C, dissociation into single cells, centrifugation of the required number at 1,500rpm for 5 mins, then re-seeding in a fresh well.

#### Generation of reporter cell lines

Generation of FOXA2-Venus reporter ESCs. FoxA2-Venus Fusion (FVF) E-85 plasmid (kind gift from Heiko Lickert<sup>51</sup>) was used as a template for amplification of the FoxA2\_exon3-venus-neo-PGK cassette, which was subsequently cloned into pGEMT-easy vector using Gibson Assembly technique. Two fragments for Gibson Assembly were amplified using the E-085 vector as a PCR template:

- 1) Fragment1 - “FoxA2\_exon3-Venus-Neo”:

GibAs\_p3\_fwd: CGGCCGCGGAATTCGATAAAGAATCAAAGACCACTGGA

GibAs\_p4\_rev: GCCTCTCATTTCTACCGGGTAGGGGAGGCGCTTTT

- 2) Fragment 2 - “3' UTR-genomic DNA”:

GibAs\_p1\_fwd: TCCCCTACCCGGTAGAAATGAGAGGCTGAGTGGAGACTTT

GibAs\_p2\_rev2: CGCGAATTCAGTAGTATCCCTCCTCCTCAATTTCTCTCCTTGTT

For CRISPR/Cas9 mediated knock-in of the Venus sequence at the 3' end of the *FoxA2* gene locus, gRNA3 (GCCTGCTAGCT CTGGTCACTG) was cloned into the pSpCas9(BB)-2A-Puro (PX459) vector, containing the Cas9 sequence and a gRNA expression cassette. For *FoxA2* locus editing, both vectors were electroporated together into the ESC cell line R1 using the Amaxa Nucleofector 4D, program CG-104. 24 hours after the electroporation, G418 (100 μg/ml) was added to the culture medium to select for positive clones. Single clones were expanded, and correct integration of the Venus cassette was validated by PCR, sequencing and standard Western blot analysis.

In brief, protein of cell lysates was obtained by directly adding 5xSample Buffer and boiling samples at 95°C for 10 mins. The protein was loaded onto a NuPAGE™ 4-12% Bis-Tris gel (Invitrogen) and run for 20 mins at 100V, then 90 mins at 150V, then proteins were blotted onto a PROTRAN nitrocellulose transfer membrane (Whatman) by TE 77 Semi-Dry Transfer Unit (Amersham). The membrane was blocked in 5% BSA in 1x Transfer Buffer (10x TB, MeOH, H<sub>2</sub>O) supplemented with Tween-20. Primary antibodies used: rabbit anti-FOXA2 (Cell Signalling Technologies, #8186S, 1:1000), and mouse anti-β-tubulin (MPI-CBG antibody facility, 1:200), HRP-conjugated secondary antibodies used: Invitrogen Goat anti-Rabbit/Mouse 1:2000.

FoxA2\_fwd5: GACCTCAAGGCCTACGAACA

Venus\_rev2: GTTGTGGCGGATCTTGAAGT (Product size: 694bp)

Generation of FOXA2-Venus and H2A-mCherry double-reporter ESCs. H2A-mCherry was integrated into the Rosa26 safe harbour locus of FOXA2-Venus cell line. We first obtained a Rosa26 targeting vector with Puromycin selection cassette (pR26 CAG AsIS/MIU, Addgene 74286, Ralf Kuehn lab,<sup>53</sup>), linearised the vector with Swal restriction digest, and used Gibson Assembly to insert the Gateway destination sequence immediately 3' to the splice acceptor site, and created a new vector termed pR26-sA-DEST. Subsequently, we performed a Gateway LR reaction between pR26-sA-DEST and pME-H2A-mCherry to create pR26-sA-H2-mCherry. To edit the Rosa26 locus, we identified a new high efficiency guide RNA sequence ACTGGAGTTGCAGATCACGA (Rosa26-gRNA1), and co-transfected p-Rosa26-gRNA1 and pR26-sA-H2-mCherry to FOXA2-Venus cells with Amaxa nucleofection. After 24 hours, cells were selected with 1 μg/ml Puromycin and individual clones were expanded. Correct integration was confirmed by Sanger sequencing and the length of the PCR amplicon from two primers:

R26\_leftout\_fwd1: GCTTGGTGCGTTTGCGGGGAT

mCherry\_rev1: TTGGTCACCTTCAGCTTGGCG (Product size: 5943bp)

### **Generation of knockout and mutant mouse ESC lines by CRISPR/Cas9**

gRNAs were designed using the following tools:

<https://portals.broadinstitute.org/gppx/crispick/public> or <https://www.vbc-score.org/><sup>56</sup>

*Shh*<sup>-/-</sup>. A short guide RNA (sgRNA) sequence (GCTGCTGGTGTGCCCCGGGCTGG) was cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid #62988) as outlined in Ran et al.<sup>52</sup> 2 μg of PX459 V2.0 containing the sgRNA sequence were electroporated into 2x10<sup>6</sup> DVI2 ESCs (HM1 background) using program A023 of the Amaxa Nucleofector II (Lonza) to ablate endogenous *Shh* gene expression. Electroporated cells were seeded onto a gelatin coated 10cm CellBind plate and cultured in 2iLIF. The following day cells were treated with 1.5 μg/mL Puromycin (Sigma) for 48 hours. Cells were grown in 2iLIF until resistant colonies were ready to be picked. Single and well spaced-out colonies were picked using a 20 μl pipette tip, dissociated in 0.25% Trypsin (Gibco) and plated onto feeder cells in serum-containing ESC medium + LIF in a 96-well plate to allow expansion. Colonies were screened and confirmed for deletions via Sanger sequencing using primers ShhKOPFW (CAAGCTCTCCAGCCTTGCTA) and ShhKOPRV (CTGCTCCCGTGTTCCTCA). The 11bp deletion caused a frameshift and premature stop codon. Cell lines were adapted back to 2iLIF conditions.

*Nog*<sup>mut</sup>. sgRNA2 (GGCGGATGTGTAGATAGTGCTGG) was cloned into the U6-IT-EF1As-Cas9-P2A-GFP vector, containing the Cas9 sequence and a gRNA expression cassette. As described previously, ESCs (R1) were electroporated with the construct using the P3 Primary Cell 4D X Kit (Lonza) and Amaxa Nucleofector 4D (program CG-104). 40 hours post electroporation, single GFP<sup>+</sup> cells were analysed and sorted based on their GFP expression relative to non-fluorescent control on a FACS Aria III machine into individual wells of 96-well plates and clonal lines were expanded. One clone was confirmed as trans-heterozygous mutant with a different sized deletion in each allele by PCR and sanger sequencing using the primers Nog\_fwd1 TGAGGTGCACAGACTTGGAT and Nog\_rev2 GCCGCCTTCCCAAGTAGA. CRISPR allele 1: large deletion followed by a frameshift and premature stop codon. 27 amino acids of NOGGIN protein remain, only corresponding to the signal peptide, followed by 21 out-of-frame amino acids. CRISPR allele 2: 33bp deletion including 8 amino acids of the signal peptide and 3 amino acids of NOGGIN.

### **Neural tube organoid (NTO) formation**

ESCs were dissociated into single cells as described in “Maintenance”. After centrifugation of the required number of ESCs in N2B27, the pellet was placed on ice then resuspended in Matrigel (Corning) to yield a suspension at 100 cells per μl of Matrigel. 45 μl of this suspension was spread evenly across a MatTek dish for FACS and RNA-seq experiments, or 4 μl of suspension was carefully distributed in the centre of an optical-bottom 96-well (PerkinElmer) for immunofluorescence experiments. Gellification was performed at 37°C for 15 mins (MatTek) or 4 min (96-well), then covered with pre-warmed N2B27 medium (2 ml per MatTek, or 100 μl per 96-well. “Standard” N2B27 medium for NTO generation was the same as the N2B27 base for 2iLIF (see “Maintenance”), with the addition of 1% sodium pyruvate (Gibco). “Advanced” N2B27 for making larger NTOs used Advanced DMEM/F-12 (Gibco 12634-010 instead of 21331-020) and was supplemented with 1% v/v non-essential amino acids (Gibco). In both cases, medium was exchanged daily from Day 2 onwards. On Day 2, 250 nM all-trans RA (Sigma) was added to the N2B27 medium, then removed after 18 hours. For perturbations to NTOs, the following proteins or small molecules were added at the concentrations indicated in the corresponding figures: BMP4 (Gibco), BMP7 (Gibco), LDN (Selleck Chemicals), NOGGIN (R&D), CHIR 99021 (Tocris), IWP-2 (Selleck Chemicals), IWR-1endo (Sigma), FGF2 (PeproTech), FGF8b (in house), or PD 0325901 (Tocris).

### **Mouse procedures**

Mouse procedures were approved under the license BMWFW-66.018/0006-WF/V/3b/2016 (“Coordination of tissue growth and patterning in neural tube development”) from the Austrian Bundesministerium für Wissenschaft, Forschung und Wirtschaft and performed in accordance with the relevant regulations. The following strains were previously described: *Sox2*<sup>CreERT2</sup>,<sup>23</sup> *Nog*<sup>Fli/+</sup>.<sup>22</sup> To conditionally delete *Noggin*, *Nog*<sup>Fli/+</sup> were bred to *Sox2*<sup>CreERT2</sup> mice to generate *Nog*<sup>Fli/Fli</sup>, *Sox2*<sup>CreERT2/+</sup> embryos. Pregnant mothers were intraperitoneally injected with 4mg tamoxifen in sunflower oil per mouse at embryonic Day E5.5. Embryos were harvested at E10.5. The expected ratios of genotypes were obtained.

## **METHOD DETAILS**

### **Immunofluorescence**

#### **Wholemount staining of NTOs**

NTOs were fixed in 2% PFA for 30 min at room temperature, by adding 1:1 volume of room temperature 4% PFA on top of culture media, to avoid Matrigel disintegration. After fixation, samples were washed three times in PBS, then blocked and permeabilised in b/p buffer (PBS, 1% BSA, 0.5% Triton X-100) for 24 hours at 4°C. Primary antibodies were applied in b/p buffer for 24-72 hours at 4°C to ensure deep penetration of the samples: LEF1 (Cell Signalling Technologies, #2230S, 1:200), FOXA2 (R&D, #AF2400, 1:400), FOXA2 (Cell Signalling Technologies, #8186S, 1:1000), NKX6.1 (DSHB, #F55A10s, 1:200). Samples were washed three times and for a total of at least 24 hours in b/p at 4°C, then incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen Donkey anti-Mouse/Goat/Rabbit 1:400) and DAPI (Sigma) for 24-72 hours at 4°C. Finally, samples were washed then stored in PBS.

The following primary antibodies were also used with modifications to the fixation and staining procedures. For SHH (Cell Signalling Technologies, #2207S, 1:300), E-Cadherin (Cell Signalling Technologies, #24E10, 1:100) and N-Cadherin (DSHB, #MNCD2, 1:50), antigen retrieval with 1x citrate buffer (DAKO) was performed for 45 mins at 65°C prior to blocking and permeabilization. For

pSMAD1/5/9 (Cell Signalling Technologies, #13820S, 1:800), fixation was performed in EMgrade PFA (Electron Microscopy Sciences) (8% diluted to a 2% final concentration) (12mins for 4 $\mu$ l Matrigel at room temperature). For both pSMAD1/5/9 and pERK (Cell Signalling Technologies, #4370S, 1:500), after fixation samples were permeabilised with methanol for 10 mins at -20°C, then blocked in db/p (PBS, 5% donkey serum, 0.3% Triton X-100) at 4°C.

#### **Optical clearing of wholemount NTOs**

PBS was removed and CUBIC-R+(N) refractive index matching solution<sup>48</sup> was added to completely cover the samples and incubated for 30-60 min at RT. After this adaptation, CUBIC-R+(N) solution was exchanged to fresh CUBIC-R+(N) supplemented with 2 mg/ml propylgallate (Sigma) to reduce photobleaching.

#### **Staining of embryo sections**

E10.5 embryos were fixed on ice for 75 mins in 4% PFA, washed in PBS, then adapted to sucrose and embedded in gelatin prior to cryosectioning. Slides were washed 3x for 15 mins each in 41°C PBS to dissolve the gelatin. Afterwards, slides underwent antigen retrieval as per SHH staining protocol above, then were blocked for 1 hour at room temperature in b/p. Primary antibodies as above were incubated overnight at 4°C in a humidified chamber. Samples were washed 3x for 5 mins with PBS+0.3% Triton X-100 at room temperature. Secondary antibodies were applied in a concentration of 1:200 in b/p and incubated overnight at 4°C. Samples were washed 3x for 5 mins with PBS+0.3% Triton X-100 at room temperature, then mounted in ProLong Diamond Antifade Mountant.

### **Imaging**

#### **Viventis lightsheet live-imaging**

ESCs were dissociated and seeded as described in “Neural tube organoid (NTO) formation”, but at a density of 80 cells/ $\mu$ l Matrigel in special Viventis chambers, lined with FEP film from the inside. Chambers were manufactured manually by gluing the FEP sheet to the plastic chambers and left to dry overnight. Chambers were cleaned with plasma to reduce hydrophobic properties and facilitate the attachment of the Matrigel. Before use, chambers were washed 3x with water and 80% ethanol, and dried and sterilised under UV light for 1 hour at room temperature.

After seeding and gellification of 40  $\mu$ l Matrigel, 1.5 ml of N2B27 medium was added to the chamber and exchanged daily from Day 2. Live-imaging was initiated from Day 3 of the NTO generation procedure, i.e. 6 hours after RA removal, over a timecourse of 48-75 hours. Samples were incubated at 37°C in 5% CO<sub>2</sub> with humidification throughout. Images were acquired using two Nikon 10x objectives (NA 0.3) for illumination and for detection one Nikon 25x (NA 1.1) water immersion objective with 18x magnification and imaged with the following settings: mCherry 50 ms exposure time, 0.5% laser power; Venus 10 ms exposure time, 3.5% laser power. Multiple NTOs at different xyz positions were imaged at each timepoint. Image acquisition was performed in stacks with 3  $\mu$ m step size every 15 mins.

#### **Spinning disk fixed sample imaging**

Wholemount NTOs and embryo sections were imaged on a Spinning Disk Confocal Olympus (inverted) microscope using a 10x (NA 0.4) air objective. Lasers were used at 100% and exposure time was adjusted to 200 ms for all lasers. Resolution was 0.65  $\mu$ m in x and y. For wholemount NTOs, 125 planes with 2  $\mu$ m spacing in z were acquired to allow for 3D reconstruction, and an automated stage was used for high-throughput acquisition with unbiased selection of multiple positions per 96-well using a grid. For embryo sections, planes with 2  $\mu$ m spacing in z were acquired. For von Mises-Fisher analyses, fixed NTO images were acquired on the same microscope but using a 40x (NA 1.25) silicon oil objective, with 0.57  $\mu$ m spacing in z.

### **Image processing and quantification**

The transition from the scattered FOXA2<sup>+</sup> state to the intermediate clustered state was scored manually in [Figures 1D](#) and [2C–2F](#). The transition is heterogeneous from NTO to NTO, and the tissue contains a high density of nuclei, so it was difficult to apply standardised parameters for automated image analysis. During manual analysis, a cluster was defined as at least three cells that were grouped together and displayed nuclear signal intensity of a minimum of 15% of Day 5 FOXA2 signal intensity.

Automated analyses were performed for [Figures 1C](#), [4C](#), [4E](#), [4F](#), [S1C](#), [S2E](#), [S3C](#), and [S4C–S4E](#) as described below.

#### **Analysis of Viventis movies**

As mentioned above, manual classification was performed to analyse data in [Figures 2C–2F](#). To avoid biases, two people analysed two different datasets and confirmed findings from two different microscope methods with the same conclusion.

For intensity quantification of clusters over time in [Figure 2D](#) (right panel), we used maximum intensity z-projections, performed intensity-based thresholding for segmentation, and quantified the total FOXA2<sup>+</sup> intensity for each cluster for individual frames with a Python script using scikit-image library. The movies were manually inspected from an orthogonal view in the x, z plane to ensure that there were no clusters overlapping in the z-direction.

For presentation, representative maximum intensity z-projections were made in ImageJ,<sup>54</sup> or movies were exported as.mp4 files.

#### **Analysis of fixed wholemount NTO images**

Raw.vsi files were processed in ImageJ.<sup>54</sup> For presentation, single representative z slices were exported as.tif files with set display ranges for each channel and without any binning in x-y.

von Mises-Fisher analysis. Imaris software was used to 3D reconstruct and visualise each NTO. FOXA2<sup>+</sup> nuclei above a set intensity threshold were identified using the spots function in Imaris and applied as a batch script. The precision of automated FOXA2<sup>+</sup> nucleus identification was checked and corrected manually. The geometric centre of the inscribed spot was considered the geometric centre of each nucleus and their x, y, z coordinates were used to calculate the concentration parameter, *k*, as the resulting vector

calculated as a sum of all vectors built from the centre of the NTO to the centre of each FOXA2<sup>+</sup> nucleus and normalised to the number of FOXA2<sup>+</sup> nuclei.

Correlation analysis of FOXA2 versus LEF1, pERK, pSMAD1/5/9 signal intensities in NTOs. To analyse the spatial correlation of FOXA2 and signalling pathways, we used images of fixed NTOs prepared and acquired on the spinning disk microscope as described above. For each NTO, we used ImageJ<sup>54</sup> to visually determine the midsagittal plane, draw a curved path with linewidth of 10 pixel within the tissue, measured the intensities of different channels along this path, and exported the results. Then, we used a Python script to calculate the Pearson correlation between the FOXA2 channel and a second channel representing LEF1, pERK, or pSMAD1/5/9. In this way, we obtained a single Pearson correlation per NTO.

3D quantification of NTO features. For 3D image analysis, we first processed the raw.vsi files to isotropic.tif files using CLIJ<sup>63</sup> GPU-accelerated image processing library in ImageJ.<sup>54</sup> Due to the “fish-bowl” effect of CUBIC-R+(N)’s high refractive index (RI = 1.5), the effective z steps were 3 μm. We therefore binned images in x-y from 0.65 μm to 3 μm so that the 3D voxel dimensions were comparable (3 μm × 3 μm × 3 μm in the x, y and z directions).

To quantify FOXA2<sup>+</sup> cluster number per NTO in 3D (Figures 4C, 4E, S4D, and S4E), we used CellProfiler 4.<sup>55</sup> First, we segmented the NTO outer boundary by applying Otsu thresholding to the z stacks from the DAPI channel. Then, we segmented FOXA2<sup>+</sup> clusters by applying a set intensity threshold to the z stacks from the FOXA2 channel and assigned these “child” clusters to their “parent” NTO. The FOXA2 intensity threshold was determined by comparison between positive control RA-treated versus negative control untreated endpoint NTOs and applied equally across all samples from the same experiment.

For scoring whether NTOs expressed any FOXA2, NKX6.1 and/or SHH (Figures 1C and S1C) or E-/N-Cadherin (Figure S2E), the mean intensity of the corresponding channel was measured inside the DAPI segmented volume. To measure the size of NTOs (Figures S4C and S4E), the NTO surface area was inferred from the DAPI segmented volume assuming spherical shape and to overcome confounding crenulation of the mask surface.

Measurement of FOXA2<sup>+</sup> cluster size and intensity. The quantification of FOXA2<sup>+</sup> cluster properties was performed using tissue cartography, which is a framework for analysing the properties of tissue configurations that can be approximated as thin surfaces. First, down-sampled multi-channel image volumes were processed in Ilastik<sup>64</sup> to produce a high contrast segmentation of NTOs from background. Surface reconstruction methods contained in the TubULAR package<sup>65</sup> were then applied to these processed image volumes to build mesh triangulations approximating the epithelial mid-surface of each NTO. FOXA2 signal intensity on each NTO was quantified by evolving the mid-surface along the positive and negative normal directions to create a set of nested “onion layers” (Figure S2H). FOXA2 signal intensity was sampled at subpixel resolution onto mesh triangles of each onion layer. Values at the mid-surface were taken to be the maximum intensity projection of the values on all onion layers projected along the normal direction. Area-weighted averaging was used to compute a single intensity value for each mesh vertex from the associated values at sample points on adjacent mesh faces.

FOXA2<sup>+</sup> clusters were identified on a per-vertex basis using a custom consensus clustering algorithm, which produces a single “best” clustering by comparing results across an ensemble of input clusterings. Prior to clustering, the FOXA2 intensity on each vertex was normalised between [0,1] by a linear re-scaling. For fixed wholemount images, this re-scaling was performed between the 5<sup>th</sup> and 95<sup>th</sup> percentiles of all raw intensities measured on all WT NTOs given the same RA treatment. For Viventis light sheet movies, this re-scaling was performed between the 5<sup>th</sup> and 95<sup>th</sup> percentiles of raw intensities from all time points measured from all NTOs visible in the same movie. A single clustering in the ensemble was generated by assigning an index to each vertex in the surface mesh using a modified k-means method. Given a set of random initial cluster centres, a modified version of Lloyd’s algorithm was used to greedily minimise the following cost function:

$$C[\{\vec{c}\}, f_B] = \frac{1}{\sum_v a_v f_v} \left( \sum_{\vec{c}_i} \sum_{\{\vec{c}\}_v} \frac{d^2(\vec{x}_v, \vec{c}_i)}{D_{max}^2} a_v f_v + \alpha \sum_v (f_v - f_B)^2 a_v f_v \right),$$

where  $\{\vec{c}\}$  denotes the set of all cluster centers,  $f_B$  is the average FOXA2 intensity of the background,  $a_v$  is the barycentric area of vertex  $v$ ,  $f_v$  is the normalised FOXA2 intensity on vertex  $v$ ,  $\vec{x}_v$  is the 3D location of vertex  $v$ ,  $d(\vec{x}, \vec{y})$  denotes the 3D Euclidean distance between two points  $\vec{x}$  and  $\vec{y}$  on the surface,  $D_{max}$  is the maximum distance between any two points on the NTO’s surface, and  $\alpha$  is a positive scalar that sets the relative weight of the foreground versus background terms in the cost function. The double sum in the first term runs over all vertices  $v$  associated to each cluster centre  $\vec{c}_i \in \{\vec{c}\}$ . The sum in the second term runs over all vertices assigned to the background. A vertex  $v$  was assigned to cluster  $\vec{c}_i$  if

$$d^2(\vec{x}_v, \vec{c}_i) < d^2(\vec{x}_v, \vec{c}_j) \quad \forall i \neq j \text{ and } d^2(\vec{x}_v, \vec{c}_i) < D_{max}^2 \alpha (f_v - f_B)^2, \text{ or alternatively to the background if}$$

$$D_{max}^2 \alpha (f_v - f_B)^2 < d^2(\vec{x}_v, \vec{c}_i) \quad \forall \vec{c}_i \in \{\vec{c}\}.$$

Intuitively, the cost function  $C[\{\vec{c}\}, f_B]$  is minimised when all points in the background have similar (low) intensities and all foreground points are assigned to a nearby cluster centre. Given an ensemble of such clusterings, the method of Rodolà et al.<sup>66</sup> was used to produce a consensus clustering that is as close as possible to all the individual clusterings in the ensemble (Fréchet sample mean of the clustering ensemble). The consensus clustering was then post-processed to ensure that each FOXA2<sup>+</sup> cluster was

connected. The use of a consensus clustering algorithm obviates the dependence of the output on the initial locations of the randomly chosen cluster centres. For given analysis,  $\alpha = 1$  was set and 10 initial cluster centres were used to generate each element of the clustering ensemble. The initial background intensity was always taken to the lowest intensity on the surface. Numerical experiments showed that the choice of  $d(\vec{x}, \vec{y})$  to be the 3D Euclidean distance produced indistinguishable results from more sophisticated methods using exact geodesic distances on the surface<sup>67</sup> after consensus clustering. Total FOXA2<sup>+</sup> surface area for a given NTO was taken to be the sum of all barycentric areas associated to each mesh vertex assigned to any FOXA2<sup>+</sup> cluster. For the quantification of FOXA2<sup>+</sup> cluster properties for fixed NTOs shown in Figure 4F, mean FOXA2<sup>+</sup> surface area per cluster was computed based on the number of FOXA2<sup>+</sup> clusters identified for each NTO.

For the Viventis light sheet movies, segmented clusters were tracked using a custom method. Care had to be taken to ensure that clusters could be tracked on the NTO surfaces as they grew and changed shape over time. NTO surfaces at subsequent time points were mapped to the unit sphere using the method of Baden et al.,<sup>68</sup> which constrains the mapping through a canonical centring of the scale factors, i.e. the ratio of the area of a local patch on the surface to the area of its image on the sphere. The final rotational degrees of freedom were registered by point matching sparse random subsets of vertices sampled from the mesh surfaces in the original data space. Numerical experiments demonstrated that more sophisticated methods involving optical flow (e.g. Lee and Kazhdan<sup>69</sup>) were not necessary. This correspondence constitutes a map  $\Phi(t) : S_t \rightarrow S_{t+1}$ , from the surface  $S_t$  at time  $t$  to the surface  $S_{t+1}$  at time  $t + 1$ , enabling us to follow the flow of material points on the surface over time. Clusters at subsequent time points were compared by measuring their mutual overlap and mutual distance. Explicitly, tracking backwards in time, the cost  $C_M$  of matching cluster  $i$  on surface  $S_t$  to cluster  $j$  on surface  $S_{t+1}$  was given by

$$C_M(i, j) = - \frac{\sigma_1 \left( \sum_v 1_{P_i}(\Phi_t(v)) + \sum_v 1_{P_j}(\Phi_t^{-1}(v)) \right)}{N_i + N_j} + \frac{\sigma_2}{2} \left( \frac{\sum_v d(\Phi_t(v), P_j)}{N_i D_t} + \frac{\sum_v d(\Phi_t^{-1}(v), P_i)}{N_j D_{t+1}} \right)$$

where  $N_{(i,j)}$  is the number of vertices in cluster  $(i, j)$ ,  $D_{(t,t+1)}$  is the average geodesic distance along the surface among all pairs of vertices on the mesh of  $S_{(t,t+1)}$ ,  $P_{(i,j)}$  denotes the surface patch corresponding to the respective cluster,  $1_{P_{(i,j)}}(x)$  is the indicator function which is equal to 1 if the point  $x \in P_{(i,j)}$  and 0 otherwise, and the  $d(x, P_{(i,j)})$  denotes the minimum geodesic distance from the point  $x$  to any point in  $P_{(i,j)}$ . The sums run over all vertices  $v$  in the respective clusters. The constants  $\sigma_1$  and  $\sigma_2$  set the relative weights of the overlap and distance terms. Intuitively,  $C_M$  is small if the images of the clusters under the flow strongly overlap. Cluster  $i$  was matched to cluster  $j$  if the cost  $C_M < C_U$ , where  $C_U$  is some fixed cost for leaving a cluster unmatched. For all NTOs, we set  $\sigma_1 = \sigma_2 = C_U = 1$ . The tracking for all NTOs was then manually checked and curated using a custom-built GUI to ensure validity.

The result of the cluster tracking described above is a directed acyclic graph (DAG) for each NTO, where nodes in the graph represent a cluster at a particular time point and edges (always pointing from a cluster at time  $t$  to a cluster at time  $t + 1$ ) describe the relationship between clusters at subsequent time points. The highly dynamic behaviour of clusters resulted in complex DAG topologies. DAGs were simplified by rejecting connected components that persisted for less than six hours and components that never achieved a minimum of 5% area fraction of the total NTO surface at any timepoint. Further simplification of the DAGs iteratively was achieved by merging simultaneous nodes in the block connected components of the corresponding undirected graph until only cut vertices remained. Branches that persisted for less than two hours simplified DAGs were rejected (Figure 2I). Extinction events that occurred during and after Day 4 were analysed. Features of both, “winning” (i.e. persisting) and “losing” (i.e. extinguished) clusters were averaged over the last hour prior to extinction (Figures 2G–2I; Table S1). Due to the high dynamicity of the clusters before this timepoint, categorisations were performed on a per NTO basis on simplified DAGs in Figure S2I.

### Analysis of embryo sections

For area measurements of FOXA2 and SHH in embryo sections, we first converted raw.vsi files to maximum intensity z-projections in ImageJ.<sup>54</sup> We set all channels to auto intensity and used the freehand selection tool from ImageJ to outline 1) the outer edge of the NT based on DAPI staining, 2) the NT lumen, 3) FOXA2 signal, and 4) SHH signal, and measured area in  $\mu\text{m}^2$ . To get the actual area of the NT, we subtracted 1) NT area by 2) lumen area. To get the normalised area measurements of FOXA2 and SHH respectively, we divided the area measurement of the signal 3) or 4) by the NT area. For presentation, maximum intensity z-projections with set display ranges were used. We analysed 6 control and 3 cKO embryos from two litters.

### RNA sequencing

RNA-seq data generated in this study are available at the Gene Expression Omnibus (GEO) under accession codes GSE221187 (FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells at Day 5, Figure 3), GSE236543 (FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells after BMP perturbations, Figure 4), and GSE260579 (RNA-seq of FOXA2<sup>-</sup> versus FOXA2<sup>+</sup> low, medium, high cells, Figure S4) and RNA-seq analyses are provided in Table S2.

### RNA-seq of RA-treated versus untreated timecourse from day 2-5

Timecourse RNA-seq data<sup>19</sup> was obtained from GEO with accession code GSE214368 (related to Figure S1). In brief, RNA-seq reads were trimmed using Trim Galore (v0.5.0), filtered to remove abundant sequences using Bowtie 2 (v2.3.4.1),<sup>57</sup> aligned to the GRCm38 genome (Ensembl release 94) using STAR (v2.6.0c) and summarised per gene with featureCounts (subread v1.6.2). Further analysis was performed using DESeq2 (v1.18.1).<sup>58</sup>

### **RNA-seq of FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells at day 5**

RA-treated NTOs were extracted from Matrigel on day 5 (related to Figure 3), dissociated into single cell suspension and FACS sorted into two cell populations based on FOXA2-Venus expression relative to non-fluorescent control on a FACS Aria III machine. For each sample, produced in three biological replicates, a minimum of 50,000 cells was sorted for subsequent RNA extraction. RNA was extracted from sorted cells using QIAGEN RNeasy Micro Kit and then underwent library preparation using the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490) and the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420), and bulk RNA sequencing on an Illumina NextSeq500 (single-end reads 75bp).

RNA-seq reads were trimmed using Trim Galore (v0.5.0), filtered to remove abundant sequences using Bowtie 2 (v2.3.4.1),<sup>57</sup> aligned to the GRCm38 genome (Ensembl release 94) using STAR (v2.6.0c) and summarised per gene with featureCounts (subread v1.6.2). Further analysis was performed using DESeq2 (v1.18.1)<sup>58</sup> and activated and inhibited upstream regulators were predicted using IPA Upstream Regulator Analysis based on the differentially expressed genes.

Differentially expressed genes (DEG) of FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> samples at a false discovery rate (FDR) threshold of 5% were subjected to IPA Upstream Regulator Analysis for predicting activated and inhibited upstream regulators. In addition, top 400 DEGs ranked by padj were used in gene set over-representation analysis with ClusterProfiler 4.6.2<sup>59</sup> and GO.db 3.16.0.

### **RNA-seq of FOXA2<sup>+</sup> versus FOXA2<sup>-</sup> cells after BMP perturbations**

NTOs were treated with RA at Day 2 for 18 hours as usual. At Day 3, i.e. 6 hours after RA removal, BMP4 (1.5 ng/ml) or LDN (100 nM) was added for 8 hours (related to Figure 4). To harvest cells, NTOs were washed twice with ice cold PBS, then dissociated in TrypLE. FOXA2-Venus<sup>+</sup> or FOXA2-Venus<sup>-</sup> cells were FACS sorted into lysis buffer in a 96-well plate, with 100 cells sorted for each sample in triplicate, then snap frozen on dry ice. RNA was extracted and sequencing libraries were prepared following the TM3' seq protocol<sup>70</sup> for subsequent quantitative sequencing with enrichment for polyadenylated RNAs. Pooled libraries were sequenced on the Illumina NovaSeq S4 PE150 (paired-end 500bp reads).

The paired-end raw reads were first split and the 12bp of UMI were extracted from Read2 with UMI-tools (v1.0.0). Reads were then trimmed with Cutadapt (v1.18)<sup>62</sup> and mapped to the mouse genome (mm10) using STAR (v2.5.2a). UMI counts at gene levels were quantified by HTSeq (v0.11.2).<sup>60</sup> The normalised counts per million (cpm) were obtained with DESeq2 (v1.26.0).<sup>58</sup>

### **RNA-seq of FOXA2<sup>+</sup> versus FOXA2<sup>+</sup> low, medium, high cells**

RA-treated NTOs were extracted from Matrigel, dissociated into single cell suspension and FACS sorted into four cell populations based on FOXA2-Venus expression relative to non-fluorescent control on a FACS Aria III machine, including Draq7 (Biosstatus) staining to exclude dead/dying cells. For each sample, produced in four biological replicates, 10,000 cells were sorted for subsequent RNA extraction. RNA was extracted from sorted cells using QIAGEN RNeasy Micro Kit and quantified and checked for integrity using the Agilent DNF 472 HS RNA Kit RNA Kit for the fragment analyser. 40ng of RNA per sample was used to generate barcoded cDNA using a one-step RT-PCR reaction according to published protocols<sup>71,72</sup> using KAPA HiFi Hot Start Ready Mix (Roche) and Maxima H Minus reverse transcriptase (Thermo Fisher). The barcoded oligo dT primers include the sequence for the TN5 adapter A. After the reaction the samples were pooled and purified using an in-house paramagnetic beads-based DNA purification kit with a ratio of 0.8X to select for full length transcripts. Barcoded oligodT primers were removed by incubation with Exonuclease 1 (Thermo Fisher) followed by enzyme inactivation. 100ng of the resulting cDNA was tagmented using Tn5 coupled to the adapter B for 10 minutes at 55°C followed by Tn5 stripping with 0.2% SDS. Illumina Nextera XT adapters were added by PCR amplification of the tagmented cDNA using the KAPA HiFi plus dNTP's kit (Roche). The library was size selected using in-house paramagnetic beads with a right side selection ratio of 0.4X and a left side ratio of 0.7X, and subsequently sequenced on the Illumina NovaSeq S4 PE150 (paired-end 150bp reads).

Paired-end raw were demultiplexed from the rest of the sequenced libraries using bcl2fastq. Then reads were further demultiplexed with BRB-seq tools using the barcoded Read1, Read2 files corresponding to the cDNA sequences provided individually for each sample. The resulting reads were trimmed for adapters and polyA using Cutadapt (v1.18)<sup>62</sup> and paired using SeqKit (v2.7.0).<sup>61</sup> Reads were aligned into the mouse genome GRCm38 as well as demultiplexed using a white list of our sample barcodes and quantified using STARsolo (v2.7.9a). Differential gene expression analysis was performed with DESeq2 (v1.42.0) (related to Figure S4).<sup>58</sup>

### **RT-qPCR**

RA-treated NTOs were extracted from Matrigel, dissociated into single cell suspension and FACS sorted into four cell populations based on FOXA2-Venus expression relative to non-fluorescent control on a FACS Aria. For each sample, produced in four biological replicates, 40,000 cells were sorted for subsequent RNA extraction. RNA was extracted from sorted cells using QIAGEN RNeasy Micro Kit and cDNA was prepared including random hexamers using the SuperScript VILO cDNA Synthesis Kit (Invitrogen), then diluted in a ratio of 1:5 for subsequent RT-qPCR analysis.

The following primers were used:

*Noggin*: fwd: TCAAAGGGCTGGAGTTCTCC, rev: TTACACACCATGCCCTCGG

*Chordin*<sup>73</sup>: fwd: CCAGAGCATCGCAGTTACAG, rev: TGTGGGATCTGTGAAACGAA

*Bmp4*<sup>74</sup>: fwd: TTCCTGGTAACCGAATGCTGA, rev: CCTGAATCTCGGCGACTTTTT

*Bmp7*<sup>75</sup>: fwd: GGCTGGCAGGACTGGATCAT, rev: GGCGCACAGCAGGGCTTGG

### Genotyping

Lysates for PCR were prepared following the HotSHOT protocol.<sup>76</sup> Briefly, yolk sacs were transferred to clean tubes and lysed in Alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA, pH 12) at 95°C for approx. 45 min. After cooling samples to 4°C, 1x volume of Neutralisation buffer (40 mM Tris-HCl, pH5) was added to stop the lysis reaction. 2  $\mu$ l of lysate was used in a 20  $\mu$ l PCR reaction. To genotype the *Nog*<sup>FL</sup>, Primers Nog F 13351 (CCA CAA TAT CCA GCC CTT GT) and Nog R 13352 (AAG AGG CCC ATG TGA GTG TC) were used to detect the floxed allele. 10  $\mu$ l PCR product was loaded onto a 2% gel and run at 110 Volt for 30 min. The WT band was 186bp, the mutant band 300bp. To genotype the *Sox2*<sup>CreERT2</sup>, primers 344 (GTCCAATTTACTGACC GTACACC) and 345 (GTTATTCGGATCATCAGCTACACC), as well as the internal control primers OIMR0042 (CTAGGCCACAGAAT TGAAAGATCT) and OIMR0043 (GTAGGTGGAAATTCTAGCATCATCC) were used. The Mutant band was 705bp, the Internal Control 324bp.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Source data for each graph are provided in [Table S1](#). Data of this study are presented as follows. Bar-plots with error bars showing standard deviation (SD): [Figures 1C, 1E, 2E, 4C, 4E, 4I, S1A, S1C, S2E, S2G, S3C, and S4D](#). Box-plots with median represented as centre line, whiskers show range of values, dots (if present) represent all measurement points: [Figures 1F, 3E, S1D, S2F, S4C, and S4E](#). Scatter dot-plots with mean represented as centre line (or dot) and error bars showing standard deviation (SD): [Figures 4F, 5C, S4G, S5B, and S5C](#). Asterisks indicate statistical significance, n.s. indicates non-significance.

**Supplemental information**

**Mouse neural tube organoids**

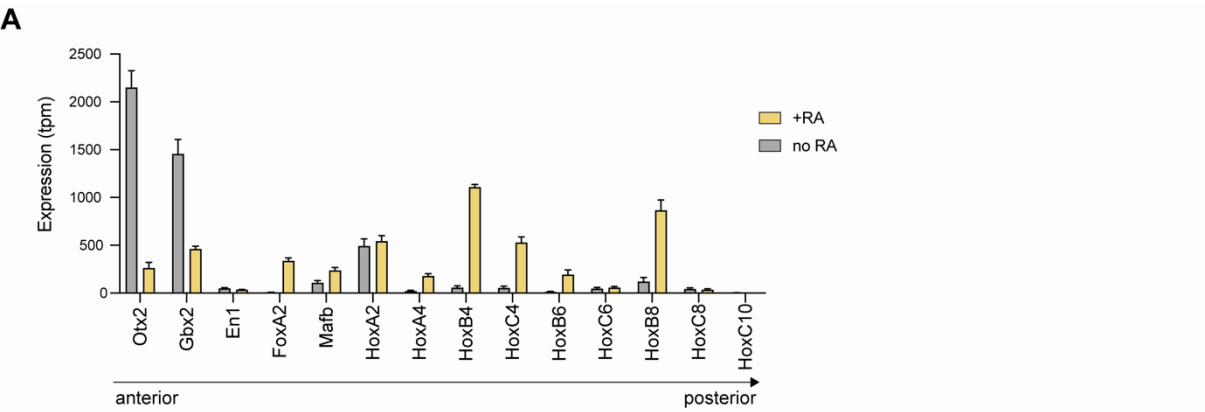
**self-organize floorplate**

**through BMP-mediated cluster competition**

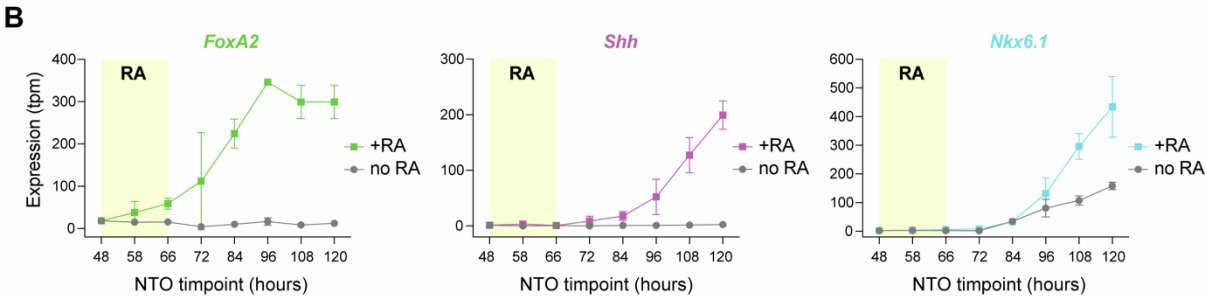
**Teresa Krammer, Hannah T. Stuart, Elena Gromberg, Keisuke Ishihara, Dillon Cislo, Manuela Melchionda, Fernando Becerril Perez, Jingkui Wang, Elena Costantini, Stefanie Lehr, Laura Arbanas, Alexandra Hörmann, Ralph A. Neumüller, Nicola Elvassore, Eric Siggia, James Briscoe, Anna Kicheva, and Elly M. Tanaka**

SUPPLEMENTARY FIGURE S1

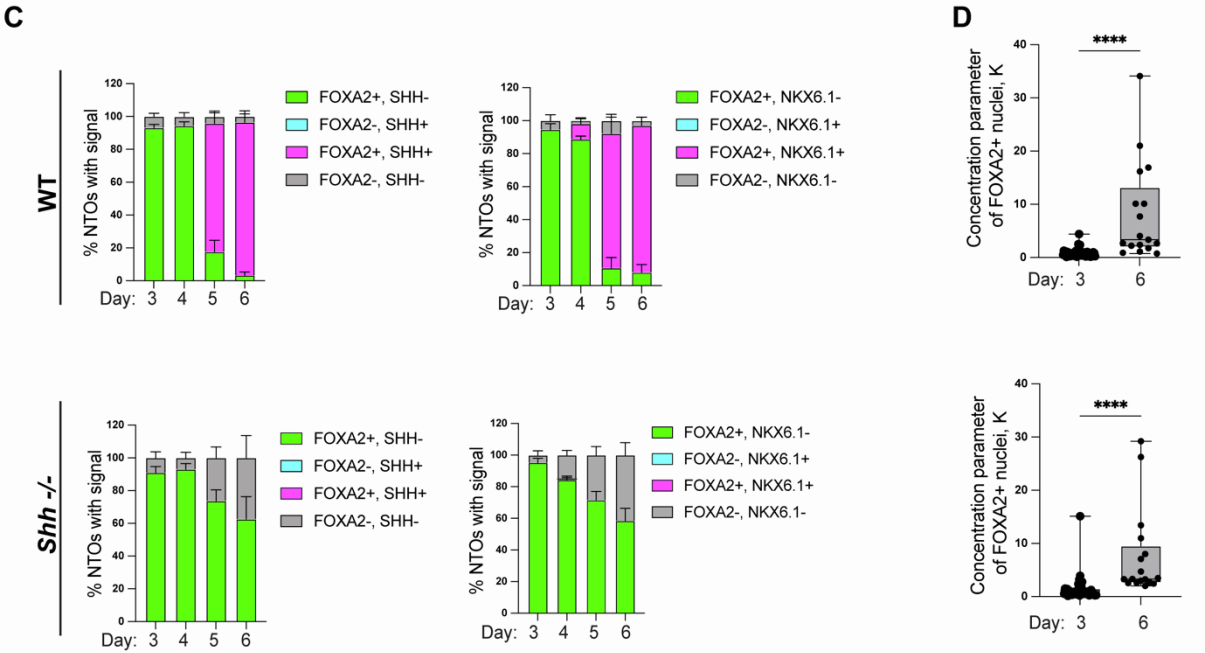
A



B



C



**Supplementary Figure S1: Characterisation of WT and *Shh*<sup>-/-</sup> NTOs.**

**A:** Bulk RNA-seq of control (no RA, grey) and RA-treated (+RA, yellow) NTOs on Day 5 shows expression levels of anterior-posterior identity markers <sup>[S1]</sup>.

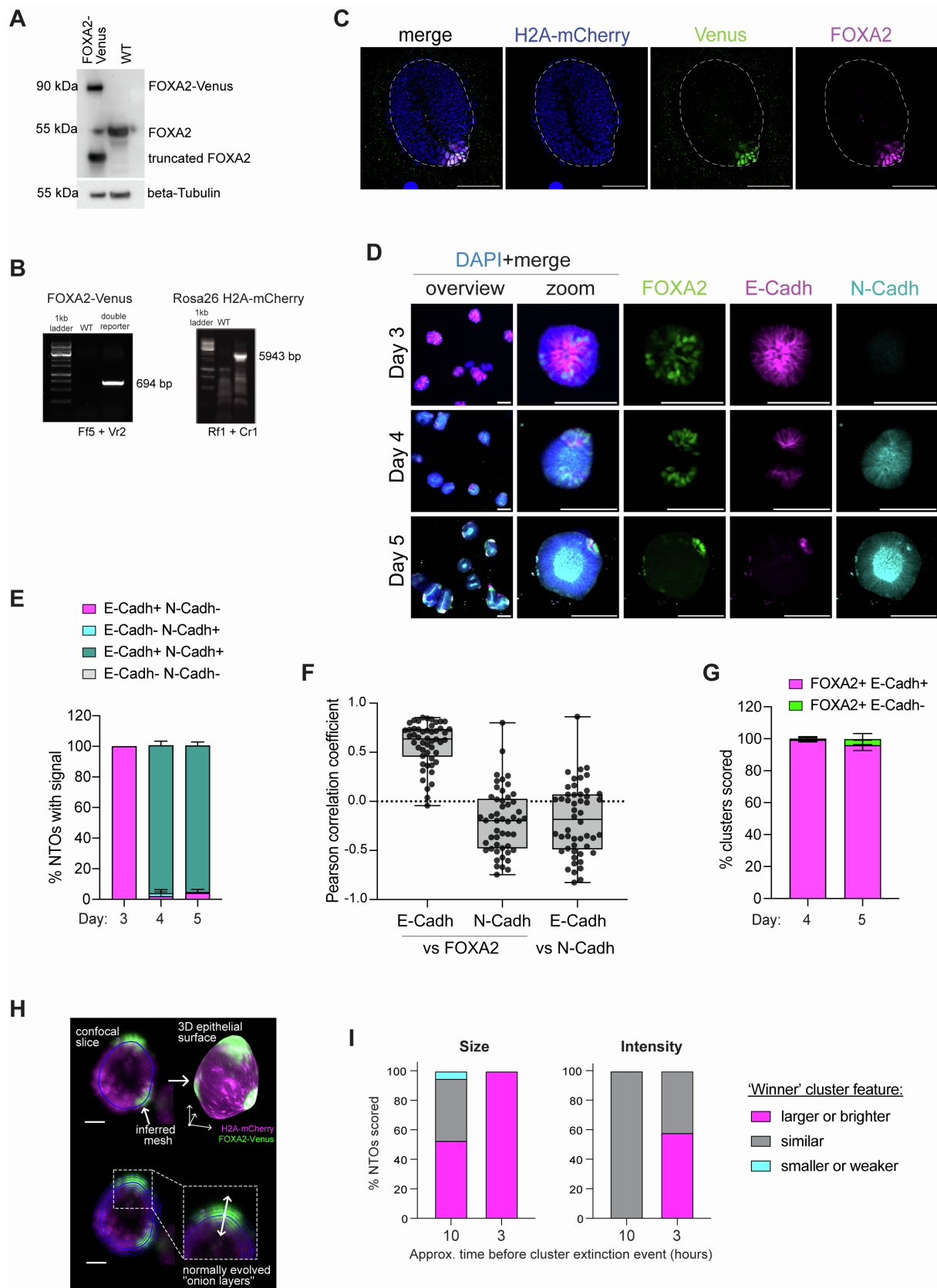
**B:** Bulk RNA-seq of NTO timecourse shows expression levels of *FoxA2*, *Shh* and *Nkx6.1* matching the colours of Figure 1B in transcripts per million (tpm) <sup>[S1]</sup>.

**C:** 3D quantification of marker expression in *Shh*<sup>-/-</sup> and parental WT NTOs over time with colours corresponding to *Shh*<sup>-/-</sup> staining in Figure 1D. *n* = 2011 NTOs for *Shh*<sup>-/-</sup> and *n* = 2239 NTOs for WT were used in the analysis of FOXA2/SHH, *n* = 1142 NTOs for *Shh*<sup>-/-</sup> and *n* = 1235 NTOs for WT were used in the analysis of FOXA2/NKX6.1.

**D:** Concentration parameter, K, measurements of *Shh*<sup>-/-</sup> NTOs and the corresponding parental WT cell line. Each dot represents the K value of a single NTO (WT: *n* = 42 for Day 3, *n* = 17 for Day 6, *Shh*<sup>-/-</sup>: *n* = 41 for Day 3, *n* = 17 for Day 6). Results were statistically analysed using a Mann-Whitney test with *p* = <0.0001. Asterisks indicate statistical significance.

**Related to Figure 1.**

## SUPPLEMENTARY FIGURE S2



**Supplementary Figure S2: Characterisation of dynamics in cluster competition of FOXA2-Venus knock-in NTOs.**

**A:** Characterisation of FoxA2-Venus knock-in line. Western Blot of FOXA2-Venus reporter line and WT detecting FOXA2 WT protein, FOXA2-Venus and a truncated FOXA2 allele. Beta-Tubulin served as a loading control.

**B:** PCR confirmation of FOXA2-Venus and Rosa26 H2A-mCherry knock in. For Venus insertion, FoxA2\_fwd5 (Ff5) and Venus\_rev2 (Vr2) (product size: 694bp), and for mCherry insertion, R26\_leftout\_fwd1 (Rf1) and mCherry\_rev1 (Cr1) (product size: 5943bp) were used.

**C:** Representative Day 5 NTO (single z slice) showing co-expression of FOXA2-Venus and endogenous FOXA2 signal with H2A-mCherry (blue), Venus (green) and stained FOXA2 (magenta). Dashed lines mark the outlines of the NTO. Scale bars, 100  $\mu$ m.

**D:** Time course of NTOs fixed from Day 3-5 (single z slice) showing a merged image at 10x magnification, and a representative NTO (zoom) stained for DAPI (blue), FOXA2 (green), E-Cadherin (magenta) and N-Cadherin (cyan). Scale bars, 100  $\mu$ m.

**E:** Quantification of marker expression in ( $n = 868$ ) NTOs over time. Coloured bars correspond to staining in A ( $n = 299$  for Day 3,  $n = 276$  for Day 4 and  $n = 293$  for Day 5).

**F:** Analysis of Pearson correlation coefficient. Intensity profiles of FOXA2 and E-Cadherin/ N-Cadherin in  $n = 50$  NTOs (Day 4) were measured and analysed by calculating the Pearson correlation coefficient by sampling the pixels for two channels (FOXA2 – E-Cadherin, FOXA2 – N-Cadherin, E-Cadherin – N-Cadherin).

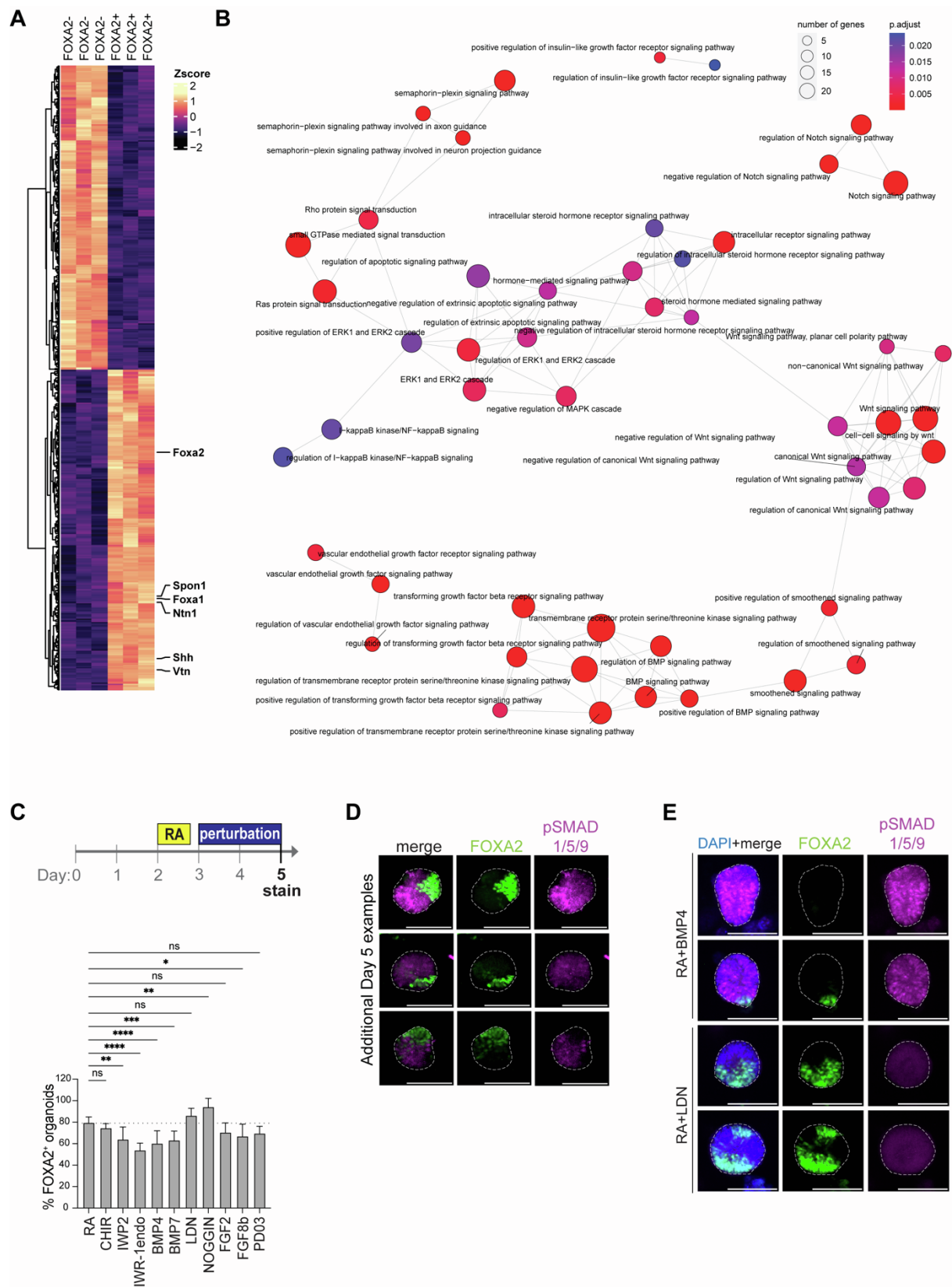
**G:** FOXA2<sup>+</sup> clusters with E-Cadherin expression. A total of 569 NTOs and 733 FOXA2<sup>+</sup> clusters were manually analysed ( $n = 267$  NTOs and  $n = 395$  clusters for Day 4 and  $n = 293$  NTOs and  $n = 378$  clusters for Day 5).

**H:** Upper panel: Schematic of the surface segmentation and fitting procedure for the construction of meshes approximation the NTO epithelial mid-surface. Lower panel: Schematic of the procedure used to measure FOXA2 expression on the surface. The surface mesh is evolved along the positive and negative normal directions to produce a set of nested “onion layers”. FOXA2 expression on the mid-surface (thick line) is taken to be the maximum intensity projection of the values on all onion layers projected onto the mid-surface along the normal direction. Scale bars, 30  $\mu$ m.

**I:** For each NTO analysed, the extinguished ‘losing’ versus persisting ‘winning’ clusters were tracked, and their properties were compared approximately 10 and 3 hours prior to the extinction event and classified into larger/ brighter, similar, smaller/ weaker.

**Related to Figure 2.**

SUPPLEMENTARY FIGURE S3



**Supplementary Figure S3: Analysis of RNA-seq data from FOXA2<sup>+</sup> and FOXA2<sup>-</sup> cells and BMP analysis.**

**A:** Heatmap of clustering of top 500 differentially expressed genes from sorted Day 5 cells.

**B:** Enrichment plots showing top 400 genes of various pathways from sorted Day 5 RNA-seq.

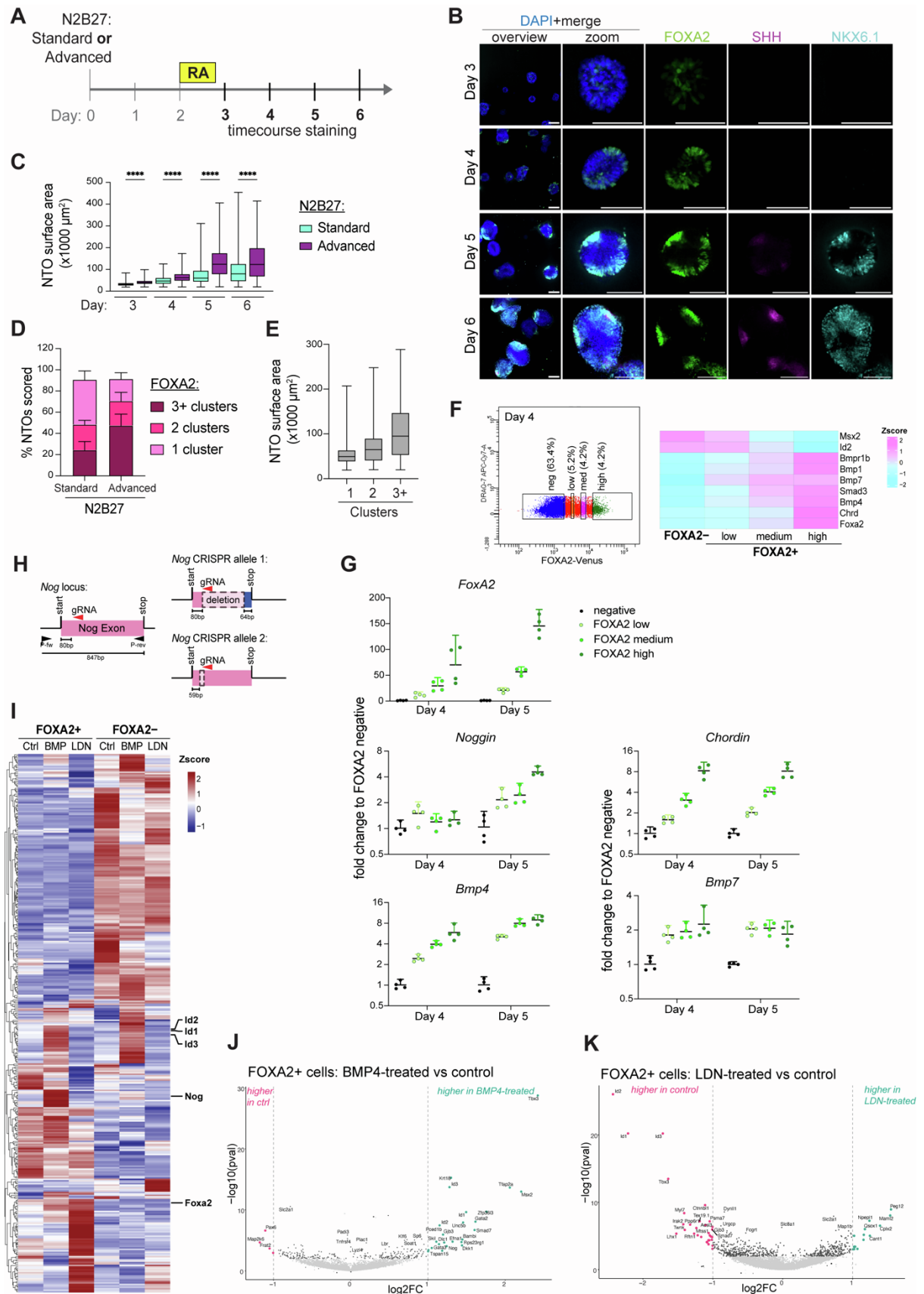
**C:** Schematic of drug screen performed on Day 3-5 starting on Day 3, i.e. 6 hours post RA removal, with analysis of proportions of FOXA2<sup>+</sup> NTOs on Day 5. The following concentrations were used: CHIR (3  $\mu$ M), IWP2 (5  $\mu$ M), IWR-1endo (1  $\mu$ M), BMP4 (5 ng/ml), BMP7 (10 ng/ml), LDN (100 nM), NOGGIN (200 ng/ml), FGF2 (10  $\mu$ g/ml), FGF8b (200 ng/ml), PD03 (1  $\mu$ M). Results were statistically analysed using an ordinary one-way ANOVA and Dunnett's multiple comparisons test. RA vs RA+IWP2  $p = 0.0019$ , RA vs RA+IWR-1endo  $p = <0.0001$ , RA vs RA+BMP4  $p = <0.0001$ , RA vs RA+BMP7  $p = 0.0009$ , RA vs RA+NOGGIN  $p = 0.0040$ , RA vs RA+FGF8b  $p = 0.0188$ , while all other tested conditions were n.s. Asterisks indicate statistical significance, n.s. indicates non-significance.

**D:** Representative examples (single  $z$  slice) of Day 5 NTOs stained for FOXA2 (green) and pSMAD1/5/9 (magenta). Dashed lines mark the outlines of the NTOs. Scale bars, 50  $\mu$ m.

**E:** Representative examples (single  $z$  slice) of Day 5 NTOs stained for DAPI (blue), FOXA2 (green) pSMAD1/5/9 to visualise BMP pathway activity (right) (magenta) upon RA+BMP4 (1.5 ng/ml), and RA+LDN (100 nM). Dashed lines mark the outlines of the NTOs. Scale bars, 100  $\mu$ m.

**Related to Figure 3.**

# SUPPLEMENTARY FIGURE S4



**Supplementary Figure S4: Characterisation of large NTOs and transcriptional induction data.**

**A:** Basic timeline of NTO production.

**B:** IF timecourse of NTOs in rich medium (“Advanced”) fixed from Day 3-6 showing (from left to right, single z slice) a merged overview image at 10x magnification, and a representative NTO (zoom) stained for DAPI (blue) and the DV patterning markers FOXA2 (green), SHH (magenta), NKX6.1 (cyan). Scale bars, 100  $\mu$ m.

**C:** 3D analysis of NTO surface area in normal NTOs (standard medium, cyan,  $n = 2463$ ) and large NTOs (advanced medium, purple,  $n = 1880$ ) from data in Figure 1B and Figure S4B. Asterisks indicate statistical significance.

**D:** 3D analysis of numbers of FOXA2<sup>+</sup> clusters in WT NTOs on Day 6 from data in Figure 1B and Figure S4B ( $n = 751$  Standard,  $n = 384$  Advanced).

**E:** 3D analysis of NTO surface area with 1, 2 or 3+ FOXA2<sup>+</sup> clusters (advanced medium, total  $n = 1321$  NTOs,  $n = 664$  for 1 cluster,  $n = 284$  for 2 clusters,  $n = 373$  for 3+ clusters).

**F:** Left: Representative plot of the gating strategy for FOXA2 levels. Right: Heatmap showing BMP pathway genes differentially expressed from BRB-seq.  $\log_{2}FC > 1$  and  $p_{adj} < 0.05$ .

**G:** RT-qPCR analysis of selected BMP pathway genes from sorted NTOs on Days 4-5. Results were statistically analysed using an ordinary one-way ANOVA and Dunnett’s multiple comparisons test, always comparing samples to FOXA2 negative of that Day (4 or 5). *FoxA2*, Day 4: neg vs low was n.s., neg vs medium was n.s., neg vs high  $p = 0.0006$ , *FoxA2*, Day 5: neg vs low was n.s., neg vs medium  $p = <0.0001$ , neg vs high  $p = <0.0001$ , *Noggin*, Day 4: neg vs low  $p = 0.0303$ , neg vs medium was n.s., neg vs high was n.s., *Noggin*, Day 5: neg vs low  $p = 0.0191$ , neg vs medium  $p = 0.0046$ , neg vs high  $p = <0.0001$ , *Chordin*, Day 4: neg vs low was n.s., neg vs medium  $p = 0.0184$ , neg vs high  $p = <0.0001$ , *Chordin*, Day 5: neg vs low was n.s., neg vs medium  $p = 0.0017$ , neg vs high  $p = <0.0001$ , *Bmp4*, Day 4: neg vs low  $p = 0.0443$ , neg vs medium  $p = 0.0003$ , neg vs high  $p = <0.0001$ , *Bmp4*, Day 5: neg vs low  $p = <0.0001$ , neg vs medium  $p = <0.0001$ , neg vs high  $p = <0.0001$ , *Bmp7*, Day 4: neg vs low  $p = 0.0299$ , neg vs medium  $p = 0.0129$ , neg vs high  $p = 0.0017$ , *Bmp7*, Day 5: neg vs low  $p = <0.0001$ , neg vs medium  $p = <0.0001$ , neg vs high  $p = 0.0006$ .

**H:** Schematic of *Nog*<sup>mut</sup> cell line generation. The gRNA binds 80bp downstream of the start codon. Sequencing of the mutant clone confirmed a trans-heterozygous mutation. CRISPR allele 1: large deletion followed by a frameshift and premature stop codon, such that only 27 amino acids of NOGGIN protein remain, which correspond only to the signal peptide, followed

by 21 out-of-frame amino acids. CRISPR allele 2: 33bp deletion including 8 amino acids of the signal peptide and 3 amino acids of NOGGIN.

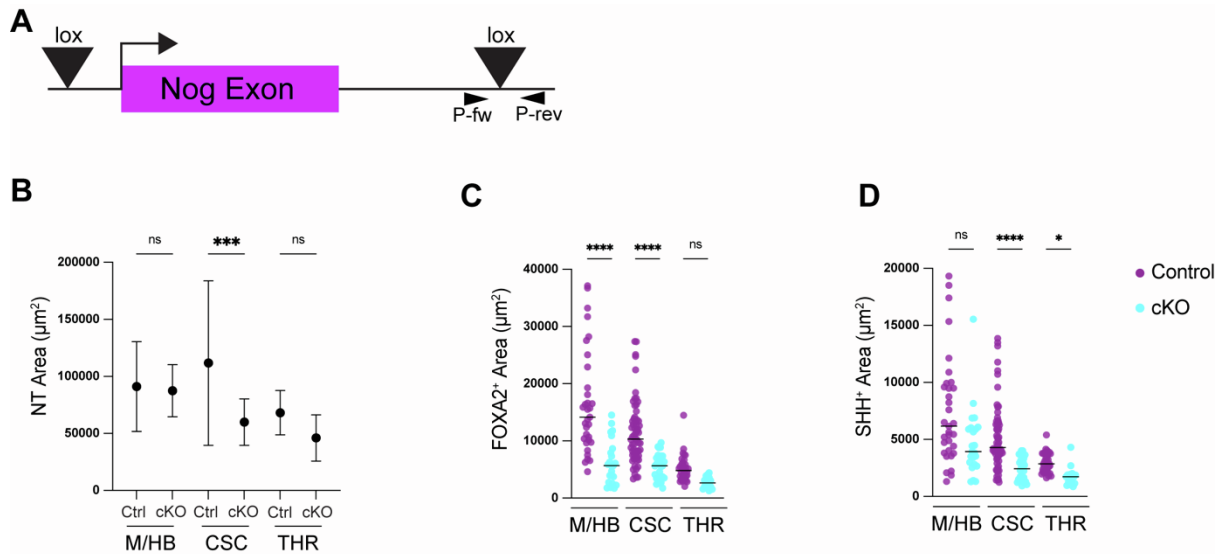
**I:** Heatmap showing clustering of top 200 differentially expressed genes from RNA-seq.

**J:** Volcano plot of genes showing Log2 fold change of expression in RA+BMP4 vs RA in FOXA2<sup>+</sup> cells from RNA-seq. Dark grey:  $p < 0.01$ , Green (#009988):  $p < 0.01$  and FC > 2, Pink (#EE3377):  $p < 0.01$  and FC < -2.

**K:** Volcano plot of genes showing Log2 fold change of expression in RA+LDN vs RA in FOXA2<sup>+</sup> cells from RNA-seq. Dark grey:  $p < 0.01$ , Green (#009988):  $p < 0.01$  and FC > 2, Pink (#EE3377):  $p < 0.01$  and FC < -2.

**Related to Figure 4.**

## SUPPLEMENTARY FIGURE S5



### Supplementary Figure S5: Characterisation of *Nog* cKO mouse.

**A:** Schematic of *Nog* flox allele and the genotyping primers.

**B:** Total area of the NT measured by DAPI signal in serial sections from the different anterior-posterior levels in of E10.5. Control (*Nog*<sup>Fl/+</sup>; *Sox2*<sup>+/+</sup> or *Nog*<sup>Fl/Fl</sup>; *Sox2*<sup>+/+</sup>) and *Nog* cKO (*Nog*<sup>Fl/Fl</sup>; *Sox2*<sup>CreERT2/+</sup>) embryos of mid/hindbrain (M/HB), Cervical spinal cord (CSC) and Thorax (THR) embryos used to normalise the data in Figure 5C. The data is represented as mean ± SD.

**C:** Raw area measurement of FOXA2 in sections from the different anterior-posterior levels corresponding to analysis from Figure 5B and C. Kruskal-Wallis test and Dunn's multiple comparison tests were performed between anatomical regions of Control vs *Nog* cKO (M/HB vs M/HB  $p = <0.0001$ , CSC vs CSC  $p = <0.0001$ , THR vs THR  $p = 0.0708$ ).

**D:** Raw area measurement of SHH in different anatomical regions from the different anterior-posterior levels corresponding to analysis from Figure 5B and C. Kruskal-Wallis test and Dunn's multiple comparison tests were performed between anatomical regions of Control vs *Nog* cKO (M/HB vs M/HB  $p = 0.1129$ , CSC vs CSC  $p = <0.0001$ , THR vs THR  $p = 0.0431$ ).

**Related to Figure 5.**

## Supplementary References

- [S1] Ishihara, K., Mukherjee, A., Gromberg, E., Brugués, J., Tanaka, E.M., and Jülicher, F. (2022). Topological morphogenesis of neuroepithelial organoids. *Nat. Phys.* 10.1038/s41567-022-01822-6.



## IV. Discussion

The primary objective of my doctoral study was to investigate the formation of a localized, fully functional floorplate in NTOs following a single global pulse of RA. I first optimized the differentiation conditions of NTOs to enhance cell viability and increase patterning efficiency. This optimization further provided in-depth insights into the epithelialization and neural differentiation trajectories of NTOs (Krammer & Tanaka, 2024). Subsequently, using this protocol, I performed live imaging, drug screening, spatiotemporal pathway analysis, and gene modification to investigate the mechanism underlying floorplate self-organization in NTOs. These experiments revealed that spatially scattered FOXA2<sup>+</sup> cells form intermediate clusters that compete with each other over long distances in a reaction-diffusion-like manner. Remarkably, this competition involves the secretion of diffusible molecules of the BMP pathway, specifically BMP4 and its inhibitor NOGGIN, from FOXA2<sup>+</sup> cells, resulting in a final floorplate. Additionally, I mutated *Noggin* in NTOs *in vitro* and conditionally deleted *Noggin* in early mouse embryos *in vivo*. These interventions respectively disrupted floorplate formation in NTOs and reduced floorplate size in the mid/hindbrain region of the mutant mouse embryos, indicating compensatory mechanisms for robustness. In my work, I demonstrate how an organizer can develop without a localized inducer, suggesting that embryonic processes traditionally considered entirely inductive may actually function through the intrinsic regulatory networks of cells.

In this part of the dissertation, I will discuss the two manuscripts that I have published in the course of my PhD and further describe open questions and potential outlooks.

### IV.1 Detailed protocols that provide thorough trouble-shooting approaches enhance the reproducibility of organoid research

#### IV.1.1 Improving the generation of NTOs

The advancement of reliable *in vitro* tools, such as neural organoids, in recent years has significantly enhanced our understanding of the formation and function of neural structures during nervous system development. The neural organoid protocol by Meinhardt and colleagues (A. Meinhardt et al., 2014) made a significant contribution by being the first clonal neural organoid protocol, mimicking early neural tube patterning. However, the efficiency of

ventral patterning was initially relatively low, and achieving optimal results was challenging. In III.1 “Neural tube organoid generation: a robust and reproducible protocol from single mouse embryonic stem cells”, I offer detailed in-depth troubleshooting strategies that enable a critical assessment at each step of the NTO protocol, which is crucial to ensure the reproducibility and reliability of experiments (Krammer & Tanaka, 2024). Gaining access to detailed protocols like this can facilitate the identification and resolution of potential issues, thereby minimizing heterogeneity and variability in experimental outcomes. This protocol set important guidelines and quality standards for generating NTOs, and additionally provides a significant and long-term contribution to reproducible research in the fields of neurodevelopment, organoids, and stem cells, which is essential to facilitate standardization across laboratories. Through the optimization of ESC culture conditions, differentiation medium, and differentiation protocol, I was able to successfully increase viability of single cells in Matrigel. This optimization subsequently enabled the use of lower input cell numbers, improved the timing of epithelialization, and increased the efficiency of ventral patterning from 40% to 80%. Following this protocol, I was able to investigate floorplate formation and ventral patterning after a single pulse of RA in NTOs in greater detail, as described in III.2 “Mouse neural tube organoids self-organize floorplate through BMP-mediated cluster competition” (Krammer et al., 2024).

## IV.2 New insights into floorplate self-organization in NTOs

### IV.2.1 Symmetry breaking and initial heterogeneity of NTOs compared to other *in vitro* systems

A global pulse of the signaling molecule RA leads to symmetry breaking and expression of scattered FOXA2<sup>+</sup> cells in NTOs that subsequently self-organize to form a coherent, fully functional floorplate (Krammer et al., 2024). How RA triggers this spatial heterogeneity of scattered expression in a defined *in vitro* system that arises through clonal expansion and epithelialization of a single cell is currently unknown. It will be important to investigate whether and how RA triggers the salt and pepper expression of FOXA2, or if an initial heterogeneity is already present in NTOs before the RA pulse is applied. The effect of symmetry breaking has been studied extensively in other *in vitro* systems such as gastruloids (Van Den Brink et al., 2014). However, this system forms from cell aggregates and not from

single cells as it is the case in NTOs (A. Meinhardt et al., 2014; Krammer et al., 2024). Gastruloids exhibit asymmetric expression of WNT, with regions of high and low WNT signaling. This asymmetry drives anterior-posterior axis elongation and induces trunk-specific differentiation on one side of the gastruloid (McNamara et al., 2023; Suppinger et al., 2023). It has been demonstrated that gastruloids can break symmetry and undergo axial elongation in well-defined hydrogel microwells, even in the absence of exogenously added WNT (Girgin et al., 2021), suggesting internal mechanisms might underly initial asymmetry induction.

It has also been suggested that early Nodal/BMP activity is sufficient to predict and control the symmetry breaking of WNT (McNamara et al., 2023). Another study reported that the asymmetric distribution of RA-dependent enzymes, which regulate its synthesis and degradation, coincides with and might cause the initial symmetry breaking in gastruloids. This affects the WNT-dependent expression of the mesoderm marker Brachyury (Hennessy et al., 2023). It will be interesting to determine whether such an initial asymmetry, caused by differential expression of metabolic enzymes, also underlies symmetry breaking and self-organization in NTOs.

#### IV.2.2 The role of physical cell-cell interactions and FOXA2-negative (FOXA2<sup>-</sup>) cells in floorplate self-organization

We performed live imaging of hundreds of NTOs to identify the mechanisms by which they form a floorplate, and found that scattered FOXA2<sup>+</sup> cells initially formed intermediate clusters that then self-organized via two non-mutually exclusive mechanisms: self-organization via cell sorting, and through long-range competition respectively cluster extinction (Krammer et al., 2024). Due to the change in cadherin expression in the course of neural differentiation (A. Meinhardt et al., 2014), and the known tendency for cells to sort based on differential cadherin expression (Tsai et al., 2020), we aimed to localize E- and N-Cadherin as potential contributors to the sorting mechanism observed in NTOs. Cadherins are transmembrane adhesion molecules and known mediators of cell-cell contacts (Maître et al., 2012), a commonly accepted mechanism of self-organization (Kay & Thompson, 2009; Krens & Heisenberg, 2011). We observed a gradual transition from E-Cadherin to N-Cadherin in FOXA2-negative (FOXA2<sup>-</sup>) cells and a striking co-localization of E-Cadherin with FOXA2<sup>+</sup> cells

during self-organization (Krammer et al., 2024). Based on these findings, we propose that cell sorting may be mediated by differential cadherin expression. Similarly, also in other *in vitro* systems, cadherins have recently been found to modulate the cellular self-organizing potential in gastruloids (Mayran et al., 2023; McNamara et al., 2023). Future research could explore the direct relationship between E-Cadherin and FOXA2 in NTOs, whether FOXA2<sup>+</sup> cells remain E-Cadherin<sup>+</sup> and use this property to move within the organoid and sort, or if E-Cadherin<sup>+</sup> cells are a pre-requisite for the persistence and clustering of FOXA2<sup>+</sup> cells. Recent advancements have demonstrated that floorplate formation *in vivo* in mouse embryos can be regulated by integrating mechanical YAP1 signaling, which is then translated into cell-specific transcriptional programs (Cheng et al., 2023). Additionally, it has been demonstrated that YAP signaling can be regulated by an atypical cadherin to induce the formation of ventral neural tube fates (Seo et al., 2022). Such a mechanism could also control the cell sorting of FOXA2 in NTOs. Moreover, it will be interesting to investigate whether the sorting of FOXA2<sup>+</sup> cells is an active mechanism or if they are passively pushed away by FOXA2<sup>-</sup> cells. Overall, understanding the role of FOXA2<sup>-</sup> cells will be crucial.

#### IV.2.3 The fate of FOXA2<sup>+</sup> cells in losing clusters during cluster competition

The other mechanism observed in NTOs for floorplate self-organization involves cluster competition facilitated by long-range morphogen signaling between intermediate FOXA2<sup>+</sup> clusters (Krammer et al., 2024). We demonstrate that self-organization does not occur randomly but through a competition between intermediate clusters that display heterogeneity in FOXA2 cluster size and brightness, thereby predicting the winning and persisting cluster. However, earlier during cluster competition these features were not as predictive, and it will be interesting in future studies to test other factors such as distance between competing clusters as potential candidates for a predictable measure of the winner cluster. Clusters losing the competition disappear, however, so far, the fate of FOXA2<sup>+</sup> cells in this scenario is not defined. It will be interesting to show if losing clusters undergo cell death or maintain but downregulate *FoxA2* expression. A potential method to address this question could be to use a FOXA2 lineage tracing approach (Delás et al., 2023).

#### IV.2.4 The requirement of SHH signaling for NTO floorplate formation

SHH fulfills many roles in inducing controlled differentiation and patterning in various cell types and tissues during vertebrate development (van Straaten et al., 1985; Placzek et al., 1990; Yamada et al., 1993; Briscoe et al., 2000; Liem et al., 2000; Martí, 2002, p. 200; Jeong & Epstein, 2003; Dessaud et al., 2007, 2008). Among other sources, SHH is secreted by the floorplate *in vivo* to pattern cell types in the ventral neural tube. Additionally, also the formation of the floorplate in the ventral neural tube in specific anterior-posterior locations depends on induction by the notochord secreting SHH (Placzek et al., 1990). Interestingly, in NTOs FOXA2 cluster formation occurs spontaneously even in the absence of SHH signaling, suggesting that SHH is not necessary for FOXA2 induction and cluster formation *in vitro* (Krammer et al., 2024). FOXA2 is expressed, self-organizes and clusters, but no ventral patterning is observed in NTOs lacking functional SHH protein. This result further promotes the hypothesis that SHH signaling is not necessary for FOXA2 expression *in vivo* (Teillet et al., 1998; Balmer et al., 2016; Sasaki & Hogan, 1993, 1994). Multiple studies in several vertebrate species have questioned the necessity for SHH signaling in floorplate induction (van Straaten & Hekking, 1991; Shih & Fraser, 1995; Patten et al., 2003).

#### IV.2.5 The long-range mechanism underlying NTO floorplate self-organization

Instead, we found that during self-organization of FOXA2 in NTOs, intermediate FOXA2<sup>+</sup> clusters do compete to promote each other's extinction, caused by the diffusible BMP pathway molecules BMP4 and NOGGIN (Krammer et al., 2024). Increase in BMP pathway activity using BMP4 leads to formation of fewer and smaller FOXA2 clusters, while NOGGIN-treatment inhibits cluster resolution and thus seems to protect FOXA2 from its downregulation. Similar to NTOs, in which BMP4-treated NTOs show increased expression of *Noggin* suggesting a feedback mechanism between FOXA2, NOGGIN and BMP to prevent BMP-dependent downregulation of FOXA2<sup>+</sup> clusters, human 2D gastruloids also reported BMP-induced *Noggin* expression (Etoc et al., 2016). Taken together, these findings create a model in which FOXA2<sup>+</sup> cells express all necessary components to drive its own self-organization into a coherent floorplate from spatially scattered cells. Due to the fact that organoid size correlates with FOXA2 cluster number, as well as increasing levels of BMP4 showing a dose-dependent reduction in NTOs with initially multiple clusters, it is likely that a

reaction-diffusion mechanism underlies observed cluster competition. To demonstrate the nature of the underlying long-range self-organization machinery, it will be crucial to localize both BMP4 and NOGGIN proteins. The ultimate demonstration of a Turing-like reaction-diffusion process would be to aim for a change in diffusivity of one of the members for example NOGGIN, by targeting it to the cells' membrane, similar to what has been done to visualize and modulate GFP gradients in *Drosophila* (Stapornwongkul et al., 2020). Furthermore, it will be interesting to investigate the temporal dynamics of the BMP4-NOGGIN-FOXA2 circuit in NTOs from subcluster (intermediate) state to clustered (final) state, to explore how FOXA2 expression levels and cluster size are controlled, and to investigate how the sorting and differential cadherin expression could be involved in the BMP-NOGGIN circuit.

#### IV.2.6 BMP signaling in the ventral neural tube *in vivo*

The role for BMP signaling during vertebrate development *in vivo* was commonly accepted as BMP inhibitors playing a crucial role in the induction of neural tissue during gastrulation (W. C. Smith & Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Sasai & De Robertis, 1997), while BMP ligands such as BMP2, BMP4 and BMP7 are mostly secreted from the roofplate and the ectoderm above to form a dorsal-ventral concentration gradient of BMP signaling to pattern the neural tube required for the induction of neural crest cells and dorsal interneurons (Basler et al., 1993; Liem et al., 1995; Lee et al., 1998; Liem et al., 2000). While research on BMPs in the developing neural tube has largely concentrated on their role in regulating dorsal cell fates, potential ventral neural cells might also encounter BMP signals either before or simultaneously with their exposure to SHH (Liem et al., 2000). It is also well known that BMP pathway inhibitors like Chordin or Noggin are expressed in the node (McMahon et al., 1998), notochord (Liem et al., 2000) and ventral neural tube (Bruneau & Rosa, 1997). Interestingly, and mostly overlooked due to their prominent role as dorsalizing factors, BMP ligands are expressed in ventral neural tissue such as notochord and floorplate (Arkell & Beddington, 1997; J. K. Dale et al., 1997; Dudley & Robertson, 1997; Furuta et al., 1997; Bruneau & Rosa, 1997; McMahon et al., 1998; K. Dale et al., 1999). BMP7 is expressed in the most anterior primitive streak that will form the node, and at the ventral midline of the anterior neural tube (Arkell & Beddington, 1997; J. K. Dale

et al., 1997) as well as in the notochord (Lyons et al., 1995; Dudley & Robertson, 1997). Maybe the most surprising fact is that upon deletion of Noggin, ectopic BMP4 expression could be observed in the notochord and ventral neural tube, and disrupted ventral patterning due to absence of floorplate cells and motoneurons at caudal axial levels (McMahon et al., 1998), without changing the range of dorsal domains into ventral sites (Arkell & Beddington, 1997; McMahon et al., 1998). This suggests that all neural tissues can express BMP ligands, but the local expression of Noggin and/ or Chordin prevents ventral neural expression under normal conditions, thus sensitizing neural cells to SHH signaling. The ventral expression of BMP pathway members agrees with the findings in NTOs, where BMPs and their inhibitors, Noggin and Chordin, are almost exclusively expressed by ventral FOXA2<sup>+</sup> cells (Krammer et al., 2024), suggesting redundant mechanisms for robustness.

#### IV.2.7 The role of other pathways potentially involved in floorplate formation in NTOs

We found that BMP signaling plays a critical role in floorplate specification *in vitro* and *in vivo*. However, previous literature has also reported a contribution of other signaling pathways to floorplate formation *in vivo* that could potentially also be important in floorplate formation in NTOs. Fate mapping experiments in zebrafish embryos have shown that Notch pathway genes are expressed in the caudal floorplate and notochord (Bierkamp & Campos-Ortega, 1993; Dornseifer et al., 1997; Westin & Lardelli, 1997; Stasiulewicz et al., 2015; Sharma et al., 2019), with many of these genes reported to play a role in midline development (Appel et al., 1999; Latimer & Appel, 2006). Notch signaling has also been shown to inhibit notochord formation and promote the proliferation, but not the specification, of floorplate progenitors in zebrafish and chick embryos (Latimer & Appel, 2006; Gray & Dale, 2010). Notch signaling also upregulates SHH expression in the floorplate in frog embryos (López et al., 2003), while its inhibition blocks FOXA2 induction by the notochord (Stasiulewicz et al., 2015). Another signaling pathway implicated in floorplate formation is Nodal signaling, which is known to be important for endoderm and mesoderm induction during gastrulation, thereby regulating left-right asymmetry in the early developing embryo. (Shen, 2007). Nodal has been reported to play a role in the induction or specification of anterior floorplate cells in several species (Placzek & Briscoe, 2005). In particular, in zebrafish embryos, initial floorplate induction is

dependent on Nodal signaling (Schauerte et al., 1998; Sampath et al., 1998; Odenthal et al., 2000; W. Chen et al., 2001; Etheridge et al., 2001; Varga et al., 2001), and blocking Nodal signaling results in inhibition of floorplate specification (Hatta et al., 1991).

It will be interesting to investigate how these pathways are regulated in NTOs and whether and how they feed into the FOXA2<sup>+</sup> self-organization system.

#### IV.2.8 NTO axial identity and correlation to *in vivo* phenotype

NTOs resemble the hindbrain/cervical spinal cord level (A. Meinhardt et al., 2014; Krammer et al., 2024), a region where clustered FOXA2 expression at the ventral midline can be observed prior to the formation of the notochord underneath (Balmer et al., 2016). The mechanisms controlling floorplate formation in this anterior region in mouse embryos were previously unknown and have been matter of debate to date (Le Douarin & Halpern, 2000; Placzek et al., 2000; Patten et al., 2003; Kremnyov et al., 2018). When we removed Noggin during early mouse development specifically in SOX2-expressing cells, we found a significant reduction of floorplate size in the mid/hindbrain region, similar to the phenotype we observed in the NTOs upon increasing BMP signaling (Krammer et al., 2024). This result further underscores the impotency of BMP pathway members in the ventral neural tube *in vivo* (Arkell & Beddington, 1997; J. K. Dale et al., 1997; Dudley & Robertson, 1997; Furuta et al., 1997; Bruneau & Rosa, 1997; McMahon et al., 1998; K. Dale et al., 1999; Liem et al., 2000), and suggests for a potential role in floorplate formation and/or maintenance in this anterior region.

#### IV.2.9 Floorplate formation in NTOs may resemble anterior floorplate induction from node-specific cells

While it is well known that the notochord is sufficient to induce the formation of a floorplate *in vivo*, there is evidence that at more anterior regions of the developing embryo, the floorplate also forms prior to notochord formation (Balmer et al., 2016). NTOs form a fully functional floorplate without receiving any spatially inductive cues from the notochord (Krammer et al., 2024). This result further questions the original view of notochord-inductive floorplate formation in mammals, and focusses on alternative models of floorplate formation

(Dodd et al., 1998). One of these models suggests that the floorplate and notochord derive from pre-specified cells of the node (I Altaba et al., 1995; Catala et al., 1996; Teillet et al., 1998; Le Douarin & Halpern, 2000; Patten et al., 2003), a structure forming during gastrulation at the anterior primitive streak at approximately E7.5 in the mouse (Downs & Davies, 1993). This timing would agree with the developmental time and stage of floorplate formation in NTOs. Mouse ESCs resemble stage E3.5 when seeded in Matrigel for differentiation into NTOs. When RA is applied two days after initial seeding, the cells resemble the E5.5 stage, corresponding to the pre-gastrulation stage. During mouse embryogenesis, RA is not detectable at E6.5 when gastrulation begins, but it becomes detectable from E7.5 onwards (H. L. Ang et al., 1996), indicating that RA signaling may start during the late primitive streak or early node stage of embryogenesis. Interestingly, mRNA of two RA receptors (RAR), RAR alpha and RAR gamma, is ubiquitously detectable at E6.5, and two metabolic enzymes capable of converting retinol to RA (B. L. M. Hogan et al., 1992; H. L. Ang et al., 1996) are expressed in the late anterior primitive streak at E7.5, coinciding with detectable RA levels (H. L. Ang & Duester, 1997; Maden, 2000). While most studies in chick embryos have shown that RA is secreted by the Hensen's node (B. L. M. Hogan et al., 1992; Y. Chen et al., 1992; Itasaki et al., 1996; Grapin-Botton et al., 1997; Muhr et al., 1999; Maden, 1999; Liu et al., 2001; Bel-Vialar et al., 2002), it has also been reported in mouse embryos that the node secretes RA (H. L. Ang et al., 1996; Niederreither et al., 1999). These findings suggest that the RA pulse applied to NTOs likely mimics RA signaling from the node, consistent with the hypothesis that RA from the node plays a critical role in anterior floorplate formation also *in vivo* (B. L. M. Hogan et al., 1992; Y. Chen et al., 1992; Itasaki et al., 1996; H. L. Ang et al., 1996; Grapin-Botton et al., 1997; Muhr et al., 1999; Maden, 1999; Niederreither et al., 1999; Liu et al., 2001; Bel-Vialar et al., 2002). This hypothesis is particularly relevant considering also the regional differences in the timing of floorplate formation compared to notochord formation and elongation *in vivo* (Balmer et al., 2016). Interestingly, chick embryos at developmental stage HH4-6 display dispersed FOXA2 expression that gradually concentrates to form a coherent cluster in midline neural explant cultures isolated anterior to Hensen's node (Patten et al., 2003), resembling the trajectory of FOXA2 expression pattern observed during floorplate self-organization (Krammer et al., 2024).

### IV.3 Final remarks

In summary, the findings of this dissertation underscore the importance of precise protocol optimization and thorough investigation of cellular behaviors in achieving high-quality, reproducible data in neural organoid research, and further provides critical insights into the self-organization mechanisms of NTOs. By elucidating the role of BMP-mediated cluster competition in floorplate formation, this work advances our understanding of neural development and the complex signaling pathways involved. Finally, the findings highlight the potential of using NTOs as robust models for studying early neural patterning and the dynamics of morphogen signaling.

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