

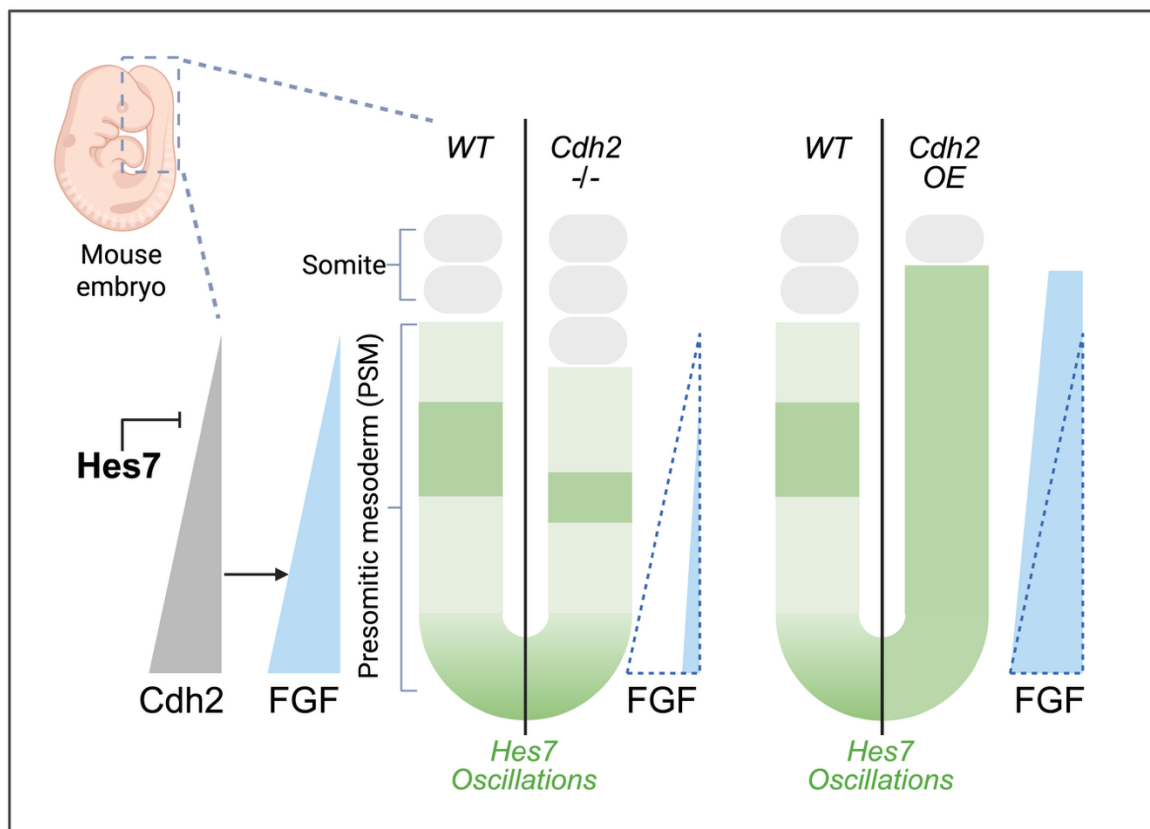
**Investigating Hes7 Regulatory Networks and
Mechanisms of Oscillatory Gene Expression During
Somitogenesis**

Xueqi JIA

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Graphical Abstract



Abstract

Embryonic development involves the transformation of a single cell into a complex organism, a process precisely guided by spatial and temporal signals. A key question is how individual cells decode these cues to decide the timing and location of their differentiation during morphogenesis. Somitogenesis in vertebrate embryos serves as a classic example of a developmental process. In this process, cells of the presomitic mesoderm (PSM) gradually progress along the posterior-to-anterior axis, differentiating in a stepwise fashion to generate somites—segmented blocks of mesoderm that ultimately form repetitive structures like vertebrae, ribs, and skeletal muscles. The segmentation clock, an oscillatory gene regulatory network, governs the periodic formation of somites. Although several core regulators, including Hes7 and ppERK, have been characterized, the mechanisms coordinating their rhythmic activity are not fully understood. Here, we employed genome editing in murine presomitic mesoderm (PSM)-like cells to identify Cdh2 as a critical mediator linking Hes7 activity to ppERK signaling. Chromatin immunoprecipitation sequencing (ChIP-seq) of HES7 revealed numerous downstream targets, among which Cdh2—encoding the adhesion protein N-cadherin—was previously unrecognized as a direct target. Functional perturbation of Cdh2 demonstrated a reciprocal regulatory relationship with Hes7 and showed that Cdh2 modulates ppERK oscillations via the fibroblast growth factor (FGF) pathway. Furthermore, appropriate Cdh2 expression was found to be essential for the timely exit of PSM cells from their undifferentiated state. These results reveal a previously unidentified role for Cdh2 in maintaining the temporal precision of the segmentation clock and another evidence of crosstalk between Notch and fibroblast growth factor (FGF) signaling during somitogenesis.

Chapter 1

Introduction

1-1 Ultradian Clocks and Their Biological Functions

Biological systems rely tightly on the rhythmic processes among a wide spectrum of time scales. Among these, the most studied case is the circadian rhythm driving a 24-hour cycle synchronizing the physiological events including wake-sleep cycle, metabolism, hormone secretion, to the environmental day-night clock (Fig.1A). The gene regulatory network involving Clock, Bmal1, Per, and Cry is intensively explored and reviewed in the context of physiological homeostasis, aging and pathological dysfunctions (Patke et al., 2020; Mortimer et al., 2025). Instead, a group of biological clocks which are shorter than 24 hours, so-called ultradian clocks, is gaining more and more attention due to its emerging significance in a variety of biological processes. Compared to the circadian clocks which are mainly coupled with environmental day-night cycles, the ultradian clocks function intrinsically to coordinate the cellular response and developmental process (Harima et al., 2014; Isomura and Kageyama, 2014; Maeda and Kageyama, 2024; Chandel et al., 2024) (Fig.1B). It is essential to understand the ultradian oscillation working mechanism for decoding how cells interpret variable stimuli and signals into decision making which is helpful for tissue regeneration and disease treatment.

The ultradian oscillations have been observed in diverse biological contexts. For example, p53 (Stewart-Ornstein et al., 2017; Wang et al., 2023) protein levels oscillate and NF- κ B (Son et al., 2023; Guo et al., 2024) exhibit nuclear translocation dynamics in response to stress, which enable cells to read out kinds of signaling inputs with temporal precision. Moreover, the ERK/MAPK signaling activity also oscillates to respond to stimuli and signaling inputs. The basis of phosphorylation dynamics enables fast and sensitive responses to precisely coordinate the heterogeneity and

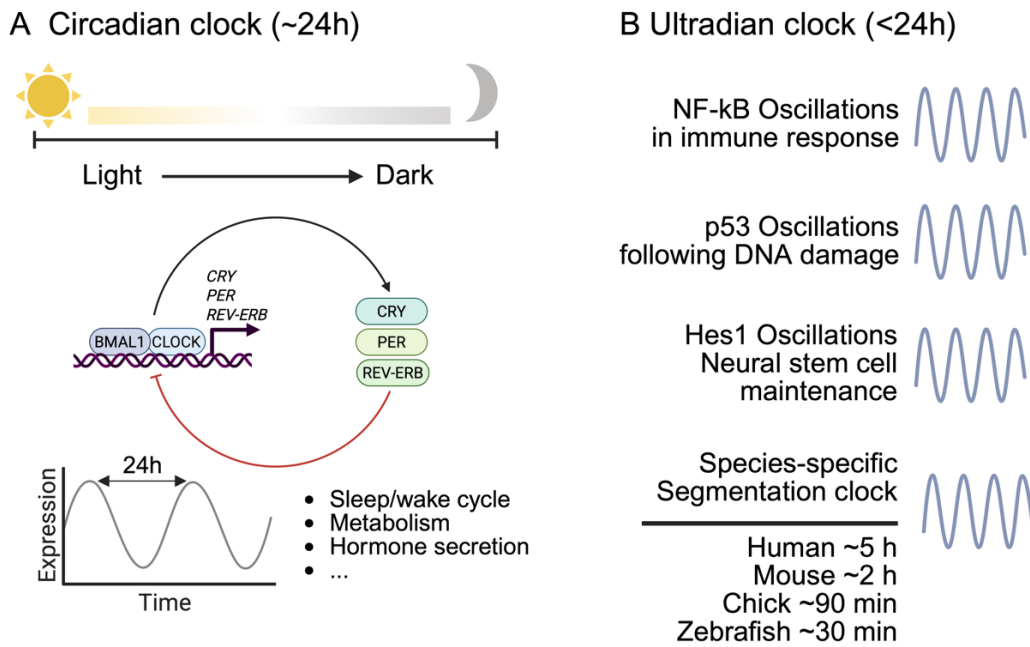


Figure 1. Examples of biological clocks. (A) Circadian clock and associated molecular oscillators. It is based on a series of interconnected positive and negative loops generated by a group of genes that show day-night cycle expression dynamics. The core regulation is composed of the binding of BMAL1 and its partner CLOCK at E-box motifs in the promoters of *PER* and *CRY* genes. After translation, the PER and CRY proteins are imported into the nucleus and inhibit BMAL1-CLOCK binding on their promoters. These cyclic regulatory loops entrain the sleep/wake cycles, metabolic activities, hormone secretion etc. (B) Ultradian oscillators in different biological contexts. NF-kB complexes shuffle between cytoplasm and nucleus in an oscillatory manner upon stimuli such as immune responses that induce IκB kinase activation. Oscillating periods vary (60min, 100min or 200min) depending on different cellular responses. The oscillation of p53 plays a key role in DNA damage responses while its periods vary across species (mouse 2.5h; human 5h). Mouse Hes1 oscillates in a ~2-hour manner to maintain neural stem cell properties. Segmentation clock is also a group of molecular oscillators with species-specific period for coupling morphogenesis.

complexity during development and other biological processes (Hiratsuka et al., 2020; Lavoie et al., 2020; Ram et al., 2023). Among the studies around stem cells, the ultradian clocks have been also strongly related to the cell proliferation, differentiation and cell fate specification. For example, neural stem cells show oscillatory expression of the transcriptional factor Hes1 where Hes1 oscillations maintain the neural stem cell renewing and the sustaining expression leads to differentiation (Shimojo et al., 2008; Imayoshi et al., 2013; Ochi et al., 2020; Maeda et al., 2023). When coupled with other essential gene expression or dynamics, Hes1 oscillations also determine neural stem cell fate specification into different lineages (Imayoshi et al., 2013, Shimojo et al., 2024). Likewise, intestinal stem cells exhibit ultradian fluctuations in expressing *Ascl1* and other regulators, coupling oscillatory gene activity to renewal and differentiation within the crypt niche (LaBella et al., 2024; Singh et al., 2024). Another case in developing pancreas, Notch components, such as *Dll1* and Hes1, and the transcription factor *NGN3* govern the balance between progenitor expansion and differentiation fine-tuning growth and the timing of endocrine lineage specification (Miller et al., 2025; Seymour et al., 2020). These findings indicate the crucial need for a much deeper understanding of the ultradian rhythm in regulating biological events.

The segmentation clock as one of the best characterized ultradian clock model in development, regulates the periodical formation of somites for building up the body trunk of the vertebrate species with species-specific periodicities (e.g., ~30 min in zebrafish, ~90 min in chicken, ~2.5 h in mouse, ~5 h in human) (Dequéant and Pourquié, 2008; Hubaud and Pourquié, 2014). In this context, genes belonging to the segmentation clock oscillate and couple with morphogen gradients to robustly translate into the temporal and spatial patterning of the future somites, which provides an ideal

system for dissecting the principles of ultradian rhythms in multicellular development (Chandel et al., 2024; Isomura and Kageyama, 2025; McDaniel et al., 2024; Miao and Pourquié, 2024).

1-2 Vertebrate Somitogenesis: A Model for Temporal and Spatial Patterning

The embryos undergo dramatic transformations in morphology where somitogenesis is one of the central events during vertebrate embryogenesis for building up the organized body axis. The vertebrate somites are the epithelial spheres of mesodermal cells derived from the presomitic mesoderm (PSM) cells sequentially. The PSM is the transient tissue that is considered originated from neuralmesodermal progenitors (NMPs) in the tailbud and located bilaterally on both sides of the neural tube along the posterior-to-anterior axis. The timing of paired somite formation is precisely regulated by a group of molecular oscillators which is called segmentation clock in the PSM (Fig.2).

The somites are not only rhythmically formed but also morphologically complicated. Each pair of newly formed somites subsequently undergo regional differentiation into different compartments of the musculoskeletal system. The ventral half of the somite becomes the sclerotome, which contributes to the future vertebrae and intervertebral discs. The dorsal compartment forms the dermomyotome, which contributes to the myotome and dermatome and produces skeletal muscle and dermis respectively. Through these transformations, somitogenesis progressively establishes the segmented vertebrate body plan for the complex structure.

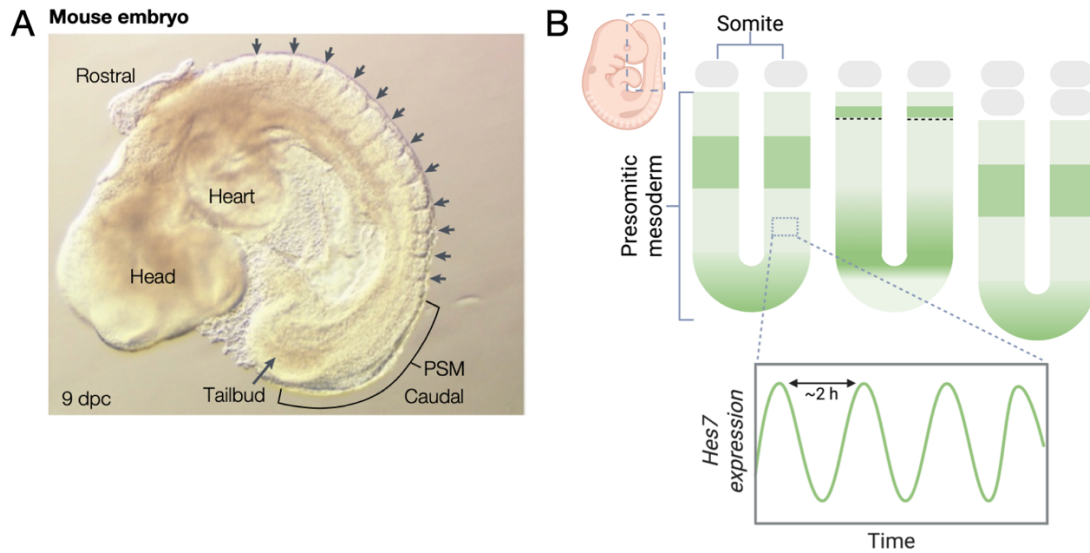


Figure 2. Illustration of mouse somitogenesis and Hes7 oscillations. (A) The representative image of 9dpc mouse embryo (image is adapted from Figure 1 by Saga and Takeda, 2001). (B) Schematic figure of mouse segmentation clock. During somitogenesis of mouse embryos, a group of genes including Hes7 exhibits oscillations where the expression of the genes starts at the posterior tip of the embryo and travels to the anterior end to coordinate the formation of a new pair of somites. Green indicates Hes7 transcriptional travelling wave. The plot illustrates the dynamics of Hes7 expression observed in the local region of PSM.

1-3 The segmentation clock and oscillatory gene expression

The concept of the segmentation clock was first proposed in the 1970s where Cooke and Zeeman suggested that the periodical formation of somites can be explained by the interactions between the molecular oscillators and the moving differentiation wavefront (Cooke & Zeeman, 1976). A few decades later, the first segmentation clock gene was revealed in the chicken where a gene called *c-hairy1* was found to exhibit oscillatory expression in the PSM tissue (Palmeirim et al., 1997). Later, its homologues in other species were also gradually revealed such as *Hes7* in mouse and *her1* and *her7* in zebrafish (Pourquié, 2011). These genes belong to the basic helix-loop-helix (bHLH) transcriptional repressors and show cyclic on-and-off expression patterns that match the somite formation periodicity. These findings established the oscillatory gene expression network that supports the molecular oscillators proposed in the model.

Among these oscillators, *Hes7* has been identified as a core component of the mouse segmentation clock (Fig.2). *Hes7* expression is specific to the PSM tissues (Bessho et al., 2001a) and oscillates with a period of about 2 hours (Bessho et al., 2001b; Hirata et al., 2004). In humans, the homologue *HES7* was found to oscillate with a 5-hour period in the PSM which is consistent with the longer time required for human embryo development (Chu et al., 2019; Diaz-Cuadros et al., 2020; Matsuda et al., 2020). Since it was uncovered, intensive genetic studies have demonstrated the critical role of *Hes7* in somitogenesis and embryo development. In the complete absence of *Hes7* expression, mouse neonates can be born; however, they exhibit severe segmentation defects, including an irregular axis and fused vertebrae, which ultimately impair lung function and result in lethality (Bessho et al., 2001b). Mutations

detected in human HES7 are associated with congenital vertebral malformation such as spondylocostal dysostosis (Lv et al., 2023; Sparrow et al., 2010, 2008). This indicates the tight relation between clock gene oscillations and body segmentation and that disruptions in this oscillatory gene expression would lead to developmental defects.

This oscillatory behavior of Hes7 relies on a transcriptional-translational negative feedback loop (Fig.3). In this loop, Hes7 protein can bind to its own promoter and repress its transcription. Due to its extremely short half-life of approximately 20 minutes (Bessho et al., 2003; Hirata et al., 2004; Matsuda et al., 2020), the Hes7 protein is rapidly degraded and consequently dissociates from its promoter, leading to the restart of the transcription. This negative feedback loop also applies to some other biological clocks, such as the circadian oscillators, however, the segmentation clock operates on much shorter timescales. Mathematically, maintaining this continuing oscillation depends on the intrinsic delays and protein turnover rates inside the loop (Lewis 2003). Experimental evidence supported the simulations where increased Hes7 protein stability with a longer half-life dampens the oscillatory gene expression and disorganizes the somite formation (Hirata et al., 2004). Moreover, removing some of the introns of Hes7 gene reduces the transcriptional delay leading to the acceleration of the oscillation tempo (Harima et al., 2013), while intron-less Hes7 results in a shorter transcriptional delay which subsequently generates steady instead of rhythmic expression and disrupts the somitogenesis (Takashima et al., 2011). These findings emphasize the segmentation clock as a well-designed system where transcription, translation and degradation steps are all paced to create stable oscillations to couple the proper timing of biological events.

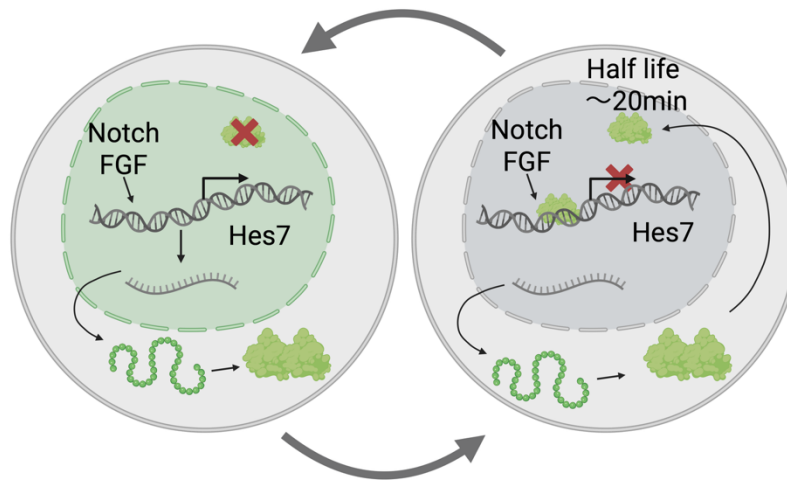


Figure 3. Illustration of the mechanism of Hes7 expression and negative feedback loop. Hes7 expression is activated by Notch and FGF pathways and its protein can bind to its own promoter to repress transcription. Since Hes7 protein has a short half-life, its transcription restarts when its protein degrades and releases the promoter regions, which forms a negative feedback loop.

Although Hes7 is central in the mouse and human segmentation clock, other genes from Notch, FGF or Wnt pathways expressed in an oscillatory manner also play important roles in this process. For example, the expression of Dusp4 and Dusp6 in mouse (Dequéant et al., 2006; Niwa et al., 2011) and zebrafish (Krol et al., 2011; Simsek et al., 2025) oscillates under the regulation by the clock and interacts with the FGF gradient activity to couple the proper PSM differentiation front. In addition, the Notch ligand DeltaC in zebrafish exhibits cyclic expression and is regulated by the her1/her7 oscillations (Oates et al., 2012), while in mice the Dll1 oscillates but is not directly related to Hes7. In chicken and mice, the expression of the *Lfng* gene is also presented in a rhythmic pattern (Aulehla and Johnson, 1999; Cole et al., 2002; Evrard et al., 1998; Forsberg et al., 1998; McGrew et al., 1998) and is a direct downstream target of Hes7 in mouse PSM (Bessho et al., 2003). With the in vitro PSC-inducible system, human LFNG is also identified as an oscillating gene in human segmentation clock (Matsuda et al., 2020). It encodes one of the members in the fringe family, LFNG, and its glycosylation activity on Notch receptors is suggested to modulate the availability of the receptor to differential ligands (Hicks et al., 2000; Kakuda et al., 2017; LeBon et al., 2014; Moloney et al., 2000). Under the repression directly by Hes7, the oscillatory expression of *Lfng* RNA in the mouse presomitic mesoderm is strongly coupled with the expression of Hes7 oscillations and the processes of periodic somite formation (Bessho et al., 2003, 2001b). On the other hand, the proper cyclic expression of *Lfng* regulates the Hes7 oscillations and somite formation (Bochter et al., 2022; Evrard et al., 1998; Okubo et al., 2012; Shifley et al., 2008; Yoshioka-Kobayashi et al., 2020). In the absence of *Lfng*, Hes7 oscillates but desynchronizes among the neighboring cells, subsequently leading to the disrupted boundaries and fused somites.

Recent studies have dissected the functional mechanism under the *Lfng*-regulated Notch signaling transduction and suggested the essential role of a coupling delay in keeping the synchrony between the neighboring cells through the post-translational modifications on Notch components by LFNG (Bochter et al., 2022; Kageyama et al., 2023; Yoshioka-Kobayashi et al., 2020). Based on an optogenetic system with light-inducible Dll1 in sender and receiver cells, this coupling delay is observed shortened in the absence of *Lfng* and longer when it is expressed, explaining that LFNG delays the Notch signal sending process and enhances the receiving amplitude. Therefore, the *Hes7* gradually oscillates out-of-phase when the essential delay of cell-cell signal transmission is shortened in the *Lfng* knockout (KO) mouse PSM, and this introduces another significant factor in understanding the biological oscillatory events.

Together, studies across species demonstrated that the segmentation clock is a group of molecular oscillators cross-talking with each other and tightly dependent on the negative feedback loop and the intrinsic-intercellular delays to maintain the systematic cyclic events. The exact operating system can differ between different species, but the core logic appears conserved. Although numerous genes have been revealed over the past few decades, there is still an urge to discover potential components to provide a more comprehensive view.

1-4 Signaling gradients and the differentiation front

The process of somitogenesis is considered as the integration of 2 core components, including the segmentation clock for providing the temporal information and the morphogen gradients for the positional cues. According to the clock and wavefront model, the integration occurs at the differentiation front, a zone within the

anterior presomitic mesoderm where the cells arrest oscillations and commit to the somite cell fate. The positioning of this differentiation front is determined by the intersection of the main morphogen gradients, FGF/ Wnt signaling descending from the posterior PSM and the retinoic acid (RA) signaling ascending from the anterior (Fig.4). As the embryo elongates along the axis, the proper distribution of the gradients moving progressively along the elongation ensures the temporal timing provided by the oscillating clocks into the spatially well-arranged repetitive structures.

The first discovery of the gradient occurred following the identification of the segmentation clock, when the FGF signalling was found to establish a posterior-anterior gradient in the mouse, chicken and zebrafish presomitic mesoderm (Dubrulle et al., 2001; Dubrulle & Pourquié, 2004; Sawada et al., 2001). The ligand *Fgf8* RNA is restrictively transcribed in the most posterior end of the embryo, and due to the progressive degradation in the earlier formed presomitic mesoderm tissue, the *Fgf8* mRNA distributed as a decreasing gradient along the posterior-to-anterior axis. This graded distribution of *Fgf8* mRNA translates into a protein gradient and subsequently activates the downstream FGF signaling effectors. The extended activation of this gradient to more anterior leads to formation of smaller somites, and the inhibition of the activity shrinks the gradient and results in forming bigger somites (Dubrulle et al., 2001; Sawada et al., 2001). This is consistent with the differentiation front proposed in the model and FGF signaling plays an important role in determining the position of boundaries.

Following the discovery of the FGF gradient, the expression of Wnt3a was also found to exhibit a posterior-anterior descending pattern, providing the evidence of a

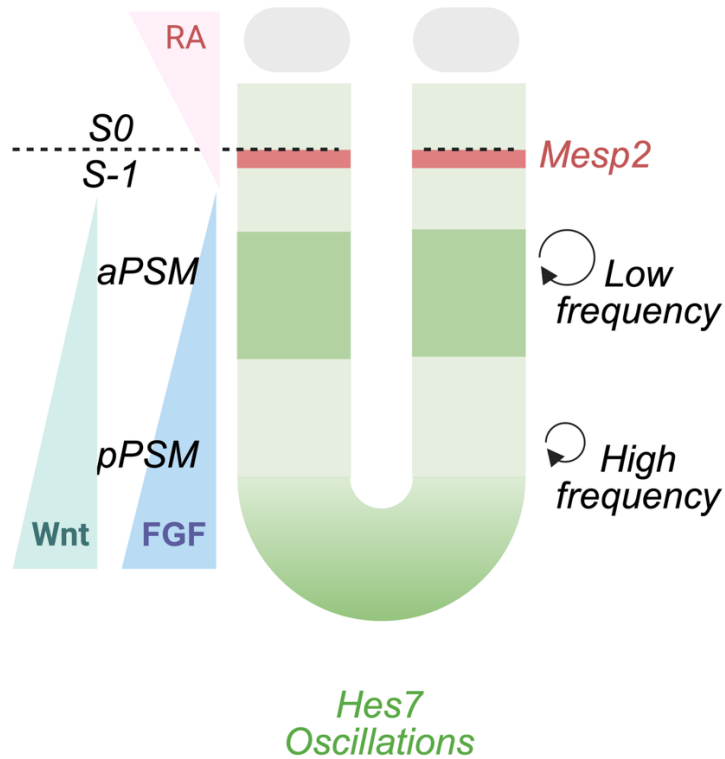


Figure 4. Signaling pathways for determining the differentiation front and PSM cells maturation. The positioning of this differentiation front which can be indicated by the expression of *Mesp2* is determined by the intersection of the main morphogen gradients, FGF/ Wnt signaling descending from the posterior PSM and the retinoic acid (RA) signaling ascending from the anterior. PSM cells at the posterior display fast oscillations and proliferative property due to high FGF/Wnt activity while as the signaling activity decays, the cells at the anterior exhibit slow oscillations and increased maturation.

Wnt signaling gradient (Aulehla et al., 2003). The absence of the Wnt3a gradient affects the clock gene activities and results in abnormal segmentation events, with broadened *Uncx4.1* expression, similar to the FGF inhibition phenotype. Moreover, reduced FGF8 expression is observed in the Wnt3a inactivation, indicating the cross talking between FGF and Wnt signaling. Oppositely, extensive activation of the Wnt signaling expanded the oscillatory fields and delayed the appearance of the *Mesp2* expression (Aulehla et al., 2008), a marker of boundary formation (Oginuma et al., 2008). Taken together, the FGF and Wnt gradients exhibit similar distribution patterns along the posterior-anterior axis and crosstalk with each other to collectively govern the boundary positioning. Thus, the posterior PSM cells are characterized by high levels of FGF and Wnt activity, which maintain the cells in an immature and oscillatory state. As the cells move anteriorly and exit the influence of these signals, the oscillations of clock gene gradually slow and eventually stop. Genetic disruption of both gradients will cause spatial misinformation for the future somite formation, resulting in severe segmentation defects.

Opposite to the FGF/ Wnt gradients, the retinoic acid (RA) signaling is observed to pose an anterior-to-posterior decreasing distribution along the axis. Its activity was suggested as antagonistic to FGF signaling (Corral et al., 2003; Moreno and Kintner, 2004) to control cell differentiation during body axis extension and to coordinate the symmetric precision of boundary position decision (Vermot et al., 2005). RA is a lipophilic molecule derived from Vitamin A and diffuses in the tissue through cell-cell membrane (Bernheim and Meilhac, 2020; Cunningham and Duester, 2015; Rhinn and Dollé, 2012). The dosage of RA is controlled through the enzymatic synthesis and degradation dynamics and regulates the gene expression during global cell patterning

and morphogenesis. RA binds to a group of RA nuclear receptors (RAR) to form heterodimer complexes to modulate the transcription of genes containing the retinoic acid response elements (RARE). Such regulatory sequences were found to be upstream of the FGF8 gene (Cunningham et al., 2015; Kumar and Duester, 2014) and in reverse the RA-metabolizing cytochrome P450, *Cyp26a1* expression requires FGF signaling activity (Boulet and Capecchi, 2012). Mouse embryos lacking expression of *Raldh2* developed bilateral asymmetric somite patterns suggesting the importance of RA signaling in keeping the segmentation clock synchrony and symmetric wave front in both lateral sides of the PSM (Vermot et al., 2005). Recent studies based on the human gastruloid systems have highlighted the requirement of RA signaling in epithelialization of the somites, indicating that besides the determination of the segmentation boundaries, a RA dose threshold might be essential for maintaining the epithelial structures during human somitogenesis (Hamazaki et al., 2024; Yamanaka et al., 2023). Thus, RA signaling establishes a differentiation permissive field by interconnecting with FGF signaling, where oscillations arrest and stable somite boundaries are specified.

Taken together, the determination front is defined by the balance point between posterior-anterior FGF/Wnt and anterior-posterior RA signaling. Cells that cross this threshold translate the temporal information from segmentation clock into spatial morphological transformation. The interplay between oscillations and gradients ensures that the cells are not only synchronized in time but also arranged precisely in space. This model is further supported by the dynamic stripe expression of *Mesp2* at the anterior end, and the precise timing for its expression onset is also in phase with the clock oscillations (Morimoto et al., 2005; Niwa et al., 2011; Oginuma et al., 2008).

Moreover, the clocks and the gradients cannot function in isolation. The crosstalk between these signaling pathways and oscillatory genes form a reciprocal feedback system. For example, inhibition of FGF signaling abolishes oscillations (Niwa et al., 2007). Instead, loss of the clock gene *Hes7* takes out the dynamics from FGF-ppERK gradients leading to altered positioning visualized by steady *Mesp2* expression and abnormal boundaries (Niwa et al., 2011). In zebrafish embryos, these phenotypes can be rescued by periodic inhibition of FGF signaling with its inhibitor (Simsek et al., 2023), demonstrating that the clock-gradient coupling is both essential and adaptable. These findings highlight that segmentation defects often arise not from the failure of a single gene but from disruption of interconnected networks.

1-5 Progress in developing in vitro models for studying the segmentation clock and somitogenesis

The segmentation clock was originally investigated exclusively in vertebrate embryos, where cyclic gene expression was visualized through in situ hybridization on fixed samples, like *c-hairy1* in chick (Palmeirim et al., 1997), *her1/her7* (Holley et al., 2000) and *Hes7* in mouse (Bessho et al., 2001a). These pioneering studies revealed the existence of traveling waves of expression in the presomitic mesoderm (PSM) in different species and opened up a new window for studying dynamic gene regulation and cell patterning. However, manipulations of embryonic systems and observations using fixed samples are often time-consuming and difficult to interpret due to pleiotropic effects. The segmentation clock oscillations in cells are dynamic and transient, so the experimental incoherency would easily mask small and subtle changes.

This was addressed since the live-imaging system with quick detection and fast turnover properties has been introduced into the somitogenesis field. First application was in the mouse embryo for visualizing the *Hes1* oscillations by using a ubiquitinated firefly luciferase reporter which shows rapid synthesis and degradations (Masamizu et al., 2006). This technique allows the in situ continuous live monitoring of the gene expression either at the tissue or the single-cell levels. Following this advance, with also the integration of the fluorescent reporter with faster maturation rates, transgenic embryos for other oscillators have been developed including mouse *Lfng* (Aulehla et al., 2008), *Dusp4* (Niwa et al., 2011) and *Mesp2* (Morimoto et al., 2005; Niwa et al., 2011), mouse *Hes7* (Yoshioka-Kobayashi et al., 2020), zebrafish *her1* (Delaune et al., 2012), and mouse *Axin2* (Sonnen et al., 2018). Coupled with advanced microscopy and computational analysis, the real-time reporter systems for fast oscillators provide a platform for measuring oscillation period, amplitude, and synchronization in greater detail, facilitating novel discoveries in a more quantitative and accurate manner. Rapid transitions or dynamic cellular behaviors can be manipulated and captured at the single-cell level. Despite these advances, the small size and limited availability of early-stage embryos hinder large-scale studies. Using intact embryos can limit throughput, reproducibility and access to certain experiments. Thus, although embryos provide physiological context, they present considerable challenges for further dissecting the molecular and dynamic properties of the segmentation clock.

To overcome these challenges, researchers have been developing and optimizing the in vitro systems using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). For the mouse somitogenesis model, the PSM-like cells were induced from ESCs through Wnt activation and BMP inhibition by mimicking the

important components in embryo presomitic mesoderm specification (Chal et al., 2015). Then the conditions were optimized by adjusting the start cell number for floating culture and applying a staged exposure to combinations of signaling factors by the order of BMP4 activation followed by Wnt activation and BMP inhibition (Matsumiya et al., 2018; Isomura et al., 2025). When cultured under these conditions, these induced PSM (iPSM) tissues exhibit robust and sustained oscillations of *Hes7* reporter, recapitulating the dynamics observed in embryos (Fig.5). Importantly, this iPSM system allows screening manipulation and live imaging in a scalable way, making it a powerful tool for discovering potential factors and mechanistic dissection for the segmentation clock. Moreover, it provided the ability to guide human iPSCs into PSM-like cell fate to study human somitogenesis which has been impossible to achieve in vivo. It revealed that human PSM-like cells oscillate at a slower period of around 5 hours, providing unique insights into evolutionary differences in developmental timing (Diaz-Cuadros et al., 2020; Matsuda et al., 2020).

Parallel to the iPSM cultures, recent advances in organoid and gastruloid technologies have further expanded the possibilities for recapitulating somitogenesis in vitro. Gastruloids were three dimensional aggregates induced from pluripotent stem cells that can spontaneously undergo gastrulation-like events and generate multi-layer axis mimicking the embryonic spatial structures (Beccari et al., 2018; van den Brink et al., 2014). By modulating the chemical and mechanical factors during the induction, the gastruloids can be guided to achieve variable morphologies, and the layer composition also can be selectively specified by adapting the protocols according to

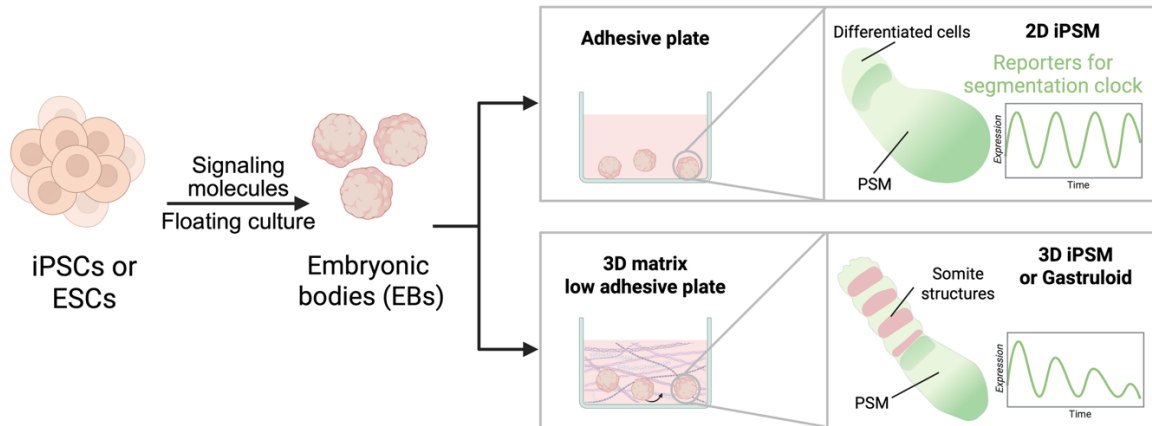


Figure 5. Scheme of in vitro somitogenesis model examples. A number of cells form aggregates when cultured in low-adhesive conditions supplemented with necessary signaling molecules for inducing presomitic lineage and differentiate into embryonic bodies containing PSM cells (iPSM). After PSM fate commitment, iPSMs can be embedded under different conditions depending on the experimental purposes. When the iPSMs were plated on the adhesive plates (e.g. fibronectin coated plates), the oscillations and travelling waves can be clearly imaged both at tissue and single-cell levels. Instead, when the iPSMs are embedded in the 3D matrix (e.g. Matrigel solution), the 3D structure can be maintained and somites formation can be observed, which is useful for dissecting morphogenetic mechanism.

specific cell preferences (van den Brink et al., 2020; Veenvliet et al., 2020). With this advancement, it is possible to recapitulate the somite-like structures in more comprehensive scale inside the dishes (Fig.5). The gastruloid protocols provides abundant selections for different research purposes as reviewed (Brink and Oudenaarden, 2021; Isomura and Kageyama, 2025; Miao and Pourquié, 2024; Sullivan and Santos, 2023) and encouraged explorations across a border range of species including human development (Hamazaki et al., 2024; Lázaro et al., 2023; Miao et al., 2023; Sanaki-Matsumiya et al., 2022; Yamanaka et al., 2023). These systems offer contexts in which the segmentation clock can be studied within a more complex, multicellular environment. These also present unique advantages where they capture self-organization processes that are difficult to reproduce in 2-dimensional cultures, and they open the door to investigating how the segmentation clock interacts with neighboring tissues such as neural tubes.

The progress in these diverse in vitro platforms creates exciting opportunities for the future experiments for dissecting the molecular and mechanistic mechanism under the regulation system of segmentation clock. By integrating CRISPR/Cas9-mediated genome editing, high resolution live imaging, single-cell transcriptomics, synthetic biological techniques and biophysical measurements into these models, researchers can dissect the segmentation clock at multiple levels from transcriptional regulation and signaling cross-talking to tissue mechanics and morphogenetic outputs. These approaches combined with traditional embryo experiments improved the availability and efficiency of embryonic studies, enabling large-scale systematic perturbations and quantitative analysis that were previously impractical. In this way,

such models are useful not only for discovering new elements but also for serving as platforms for testing new hypotheses.

1-6 Knowledge gaps and aims of this thesis

Despite major advances in our understanding of the mouse segmentation clock up to date, many questions still remained unresolved. It is still unclear how the cells keep collective synchrony which is one of the key properties in PSM oscillations for building up body plan. Cell-cell communications, in particular Notch signaling are essential for the synchronized oscillations in the population (Hubaud et al., 2017; Masamizu et al., 2006), however detailed molecular and mechanistic mechanisms still require more exploration. As summarized above, *Hes7*, the master clock gene and a transcriptional repressor in mouse segmentation clock, is indispensable to precisely pace somite formation. While its oscillatory mechanism has been well elucidated, our coverage of its downstream effectors and the whole regulatory network is still incomplete. Previous work has demonstrated that the *Hes7* target, such as *Lfng*, provides feedback to modulate Notch signaling and thereby sustain synchronized oscillations (Okubo et al., 2012; Yoshioka-Kobayashi et al., 2020). However, it remains unclear whether other *Hes7* targets also contribute to oscillatory robustness, and if so, how they mechanistically couple transcriptional oscillations to morphogen gradients and cell behaviors within the PSM tissues.

The overall aim of the study presented in this thesis is to clarify the downstream regulatory network of *Hes7* and its contribution to segmentation clock dynamics. Specifically, I sought to map the direct targets of *Hes7* in mouse presomitic mesoderm using Chromatin immunoprecipitation sequencing (ChIP-seq). By taking advantage of

the newly developed in vitro iPSM models and gene editing techniques, I evaluated the functional importance of selected genes and investigated in detail the role of the potential effectors in regulating segmentation clock. Cdh2 emerged as a critical factor influencing oscillatory dynamics. This discovery that Cdh2 is directly repressed by Hes7 raised the intriguing possibility that cell adhesion molecules are functionally integrated into oscillatory networks, linking the molecular clocks to the structural and signaling properties of the PSM tissue. By combining CRISPR/Cas9-cased gene loss of function and inducible gene gain of function strategies with live imaging, I aimed to determine how Cdh2 is involved in Hes7 oscillations and tissue organization, and to uncover how its interaction with FGF/ERK signaling links the clock and FGF gradient to sustain rhythmic oscillations and regulate the timing of PSM cell differentiation. Ultimately, this thesis seeks to place these findings within the broader framework of segmentation clock regulatory network, revealing how adhesion molecules can act as integrators of transcriptional oscillations, signaling gradients and morphogenetic processes.

Chapter 2

Materials and Methods

2-1 Mouse embryonic stem cell culture and maintenance

2-1-1 Mouse embryonic stem cell

Mouse embryonic stem cells (mESCs) employed in this study were derived from the E14tg2a line, obtained from the RIKEN BioResource Center (strain ID: RBRC-AES0135). A reporter cell line (referred as 6E6) was derived from the E14tg2a background. In this line, a DNA construct carrying the fluorescent protein Achilles under the control of the *Hes7* promoter was stably integrated into the genome, enabling real-time visualization of *Hes7* transcriptional activity (Isomura et al., 2025). This reporter was used throughout the present study for monitoring segmentation clock oscillations in live cells.

2-1-2 Cell maintaining

For routine culture, cells were kept under the a2i condition, previously described to promote stable self-renewal and pluripotency of mESCs (Choi et al., 2017; Yagi et al., 2017). Specifically, NDiff227 basal medium (Takara Bio, Y40002) was supplemented with the GSK3 β inhibitor CHIR99021 at a final concentration of 3 μ M (Sigma, SML1046), together with 1.5 μ M of the Src family kinase inhibitor CGP77675 (Cayman, 21089). To sustain pluripotency, the medium was further enriched with 1,000 U/mL leukemia inhibitory factor (LIF; StemSure LIF, FUJIFILM Wako, 199-16051). Standard antibiotics were included for contamination control, consisting of 100 U/mL penicillin and 100 μ g/mL streptomycin (Nacalai Tesque, 09367-34).

Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO₂, and their conditions were examined daily under a phase-contrast microscope to confirm uniform colony morphology, characterized by compact, dome-shaped colonies with smooth edges. When cultures reached approximately 70–80%

confluency (typically after 2 days), cells were dissociated with Accutase and reseeded at a density of 1×10^5 cells per well in a six-well plate to maintain growth and minimize spontaneous differentiation. Passaging was routinely carried out every two days to maintain stable growth kinetics and preserve pluripotent characteristics.

2-2 Cell line establishment

2-2-1 Plasmid construction

Plasmid constructs used in this study were generated using standard molecular cloning approaches, including PCR amplification, restriction enzyme digestion, ligation or Gibson assembly, and bacterial transformation, followed by sequence validation.

DNA fragments corresponding to target sequences were amplified by PCR using KOD Plus DNA polymerase (Toyobo, KOD-211) with the forward and reverse primers designed to ensure high fidelity of product generation. For shorter inserts, such as sgRNA sequences, double-stranded fragments were generated by annealing pairs of complementary oligonucleotides, followed by ligation with T4 DNA ligase (Takara, 2011A).

Backbone plasmids were linearized by restriction enzyme digestion. Digestion solution contained 0.5–1 μ g template plasmid, 1 $\times$ rCutSmart buffer (NEB #B6004), and the appropriate restriction enzymes. Samples were then incubated at 37 °C for 1 h, followed by treatment with CIAP (Takara, 2250A) to prevent re-ligation. The reaction was incubated for an additional 30 min before analysis by agarose gel electrophoresis. Linearized backbone bands were checked and purified for further steps.

Purified insert and backbone fragments were ligated using either T4 DNA ligase (Takara, 6023) or Gibson assembly. For conventional ligation, reactions were prepared

in an insert-to-vector ratio of 3 to 1, mixed with 2× ligation buffer and incubated at 16 °C for >30 min. For Gibson assembly, fragments were combined using the NEBuilder HiFi DNA Assembly kit (NEB, M5520AVIAL) at optimized molar ratios, incubated at 50 °C for 15–50 min, and placed on ice until transformation. Insert-to-vector ratios were calculated using the NEBioCalculator tool to maximize assembly efficiency.

Assembly reactions were introduced into *Escherichia coli* DH5α competent cells. Briefly, 50 µL of thawed competent cells were mixed with ligation products on ice for 20 min, heat-shocked at 42 °C for 45 s, and placed back on ice for 2 min. After adding 1 mL antibiotic-free LB medium, cultures were incubated at 37 °C for 30 min. Cells were pelleted by centrifugation (8,000 rpm, 1 min), resuspended in 150 µL LB, and plated onto LB agar plates containing ampicillin. Plates were incubated overnight at 32–37 °C.

Individual colonies were picked and expanded in LB medium for miniprep DNA isolation using a standard plasmid purification kit (Invitrogen™, K210011). Constructs were validated by Sanger sequencing (RRD DNA sequencing service). Data were analyzed to confirm sequence integrity prior to experimental use.

2-2-2 CRISPR/Cas9-mediated gene knockout plasmid design and sgRNA selection strategy

All constructs were generated using the protocol described as above. The plasmid backbone was derived from the Tol2 transposon system, originally developed by Kawakami and colleagues (Kawakami, 2007; Yagita et al., 2010). For CRISPR-based mutagenesis, we employed the high-fidelity nuclease eSpCas9(1.1) (Addgene plasmid #71814), which reduces off-target cleavage compared to conventional SpCas9 (Ran et al., 2013; Slaymaker et al., 2016). The nuclease was expressed from

a modified plasmid in which an eSpCas9(1.1)-P2A-puromycin resistance cassette was placed under the control of the human pEFS promoter. In parallel, single guide RNAs (sgRNAs) were transcribed from a glutamine tRNA promoter, as described previously (Mefferd et al., 2015). Candidate sgRNA sequences were introduced into the expression vector via the restriction site BsmBI, enabling seamless cloning of custom guide sequences.

The selection of sgRNA target sites followed a multi-step computational pipeline to maximize efficiency and minimize off-target editing. First, putative candidate sequences were generated using inDelphi, which predicts repair outcomes and frameshift frequencies at defined genomic positions (Shen et al., 2018). Among these candidates, guides predicted to generate high levels of frameshift mutations were prioritized. To further ensure specificity, sequences with potential off-target sites in the mouse genome were excluded by cross-referencing with the CRISPOR platform (Concordet and Haeussler, 2018). From the filtered set, the final sgRNA for each gene was chosen with the highest score based on a predicted activity from DeepSpCas9, a deep-learning model optimized for eSpCas9(1.1) and glutamine-tRNA promoter-driven expression (Kim et al., 2019). For negative controls, sgRNA sequences validated in previous studies to lack functional genomic targets were used (Joung et al., 2017).

1	sgRNA as negative control	GCGAGGTATTTCGGCTCCGCG
2	sgRNA for <i>Scarf2</i> -KO	GTCCCAGCCCTCGGGCTCCC
3	sgRNA for <i>Smoc1</i> -KO	GTGCAGCTGTCCCCGGCCGG
4	sgRNA for <i>Lfng</i> -KO	CAGCCTGGACAGGCCCATCC
5	sgRNA for <i>Hes7</i> -KO	GGTGGAAGTACCTGGTCCC
6	sgRNA for <i>Irf2bp1</i> -KO	CGACAGTGGCGGTGGCTTGG
7	sgRNA for <i>Kpnb1</i> -KO	ACACACTGTGAGGCCGAAGT
8	sgRNA for <i>Tmem131l</i> -KO	GGGCTCCGGCGCCCGCAGTC
9	sgRNA for <i>Khdrbs1</i> -KO	GGAACCCACCACCGTCGCGT
10	sgRNA for <i>Trim71</i> -KO	GTTCTTGGGCGGCGGCTCTT
11	sgRNA for <i>Foxo3</i> -KO	GAAGGCCCCCCGGCGGCGTG
12	sgRNA for <i>Jak3</i> -KO	TGTCATAGTCCTGCGGGCTG
13	sgRNA for <i>Cdh2</i> -KO	TCCGCGGCGCTCCCGCTATC
14	sgRNA for <i>Bcl2l11</i> -KO	GTTCTGTCTGTAGGGAGGTA
15	sgRNA for <i>Pcbp1</i> -KO	GGTGACGGACTGCGGCACGC
16	sgRNA for <i>ler5</i> -KO	AGGCTCGGGCTCCGGCCAGG
17	sgRNA for <i>Rab34</i> -KO	GCCTGCCAGGAGCACCGGAC
18	sgRNA for <i>Eno3</i> -KO	GACTCTAGGTCCTGCTCTGC
19	sgRNA for <i>Tead4</i> -KO	CCTTGCCACGGAGGAGCGAG
20	sgRNA for <i>Cited1</i> -KO	AAGCTTCTGAAGCTGCATGC
21	sgRNA for <i>Tra2b</i> -KO	ATTCTCCCATGTCTACTCGA
22	sgRNA for Cdh2-mScarlet KI	ACTGAACGGCAGGACGGACT
23	sgRNA1 for Cdh2 ECD4 deletion	AGCAGTGAGCGTGGTCAGCA
24	sgRNA2 for Cdh2 ECD4 deletion	TATCAATGACAACGCCCTC

Table 1. List of sgRNA sequences used for mutagenesis in this thesis.

2-2-3 Overexpression Systems and Inducible Constructs

For gain-of-function studies, inducible overexpression vectors were generated using a Tet-On system. The backbone contained two expression cassettes: one in which the reverse tetracycline-controlled trans-activator rtTA-3G was linked to a puromycin resistance marker via a self-cleaving P2A peptide under the control of the pCAGG promoter, and a second cassette in which the tetracycline-responsive TRE-3G promoter drove expression of nuclear-localized mCherry (NLS-mCherry).

To construct plasmids for candidate gene overexpression, full-length coding sequences (CDS) of *Hes7* or *Cdh2* were cloned into the TRE-3G cassette upstream of the NLS-mCherry marker. Each insert was fused to an HA epitope tag followed by a P2A sequence, ensuring that the transgene and mCherry were expressed independently from the same transcript. This design allowed fluorescent detection of transgene-positive cells while simultaneously maintaining functional protein expression.

In parallel, an “empty” construct lacking the *Hes7* or *Cdh2* insert was assembled and used to generate negative control lines, enabling direct comparison with experimental overexpression cell lines.

2-2-4 Generation of Cdh2-mScarlet Knock-In Cell Lines

To visualize endogenous Cdh2 protein dynamics, a donor vector was engineered for targeted insertion of the fluorescent protein mScarlet at the C-terminal end of the *Cdh2* coding sequence. The donor construct was generated using the Bluescript II SK(–) backbone, into which the mScarlet cDNA was inserted. To ensure precise homologous recombination, the fluorescent tag was flanked by ~1,000 bp

sequences corresponding to genomic regions immediately upstream (5' homology arm) and downstream (3' homology arm) of the *Cdh2* stop codon.

For positive selection of successfully transfected cells, a cassette containing pEFS-iPFP-P2A-Neomycin was integrated downstream of the 3' homology arm. This design allowed enrichment of knock-in clones through neomycin resistance, while also providing a fluorescent reporter (iPFP) for tracking transfected populations.

2-2-5 Transfection and Selection of Stable cell Lines

Reporter mESCs (6E6, Isomura et al., 2025) were transfected with plasmid constructs following previously established procedures (Isomura et al., 2025; Maeda et al., 2023). A DNA–lipid complex was prepared by mixing 0.5 µg of the designed plasmid with 0.5 µg of the helper plasmid pCAGGS-mT2TP and 3 µL ViaFect transfection reagent (Promega, E4981), diluted in Opti-MEM™ (Gibco, 31985-070). The transfection mixture (100 µL) was combined with 2×10^5 cells suspended in 100 µL a2i medium and plated into 12-well dishes. After 48 hours, cultures were subjected to puromycin selection (1 µg/mL) in a2i medium to enrich for successfully integrated clones. Resistant populations were subsequently passaged at low density to facilitate single-cell isolation (Fig. 6B). Genotyping of frameshift mutations was validated by sequencing to detect indels at the cut site, and clones with confirmed frameshifts in both alleles were selected. For each gene, at least three independent clonal lines were established for downstream analyses.

To generate knock-in lines expressing *Cdh2* fused to mScarlet, cells were first selected in a medium containing 200 µg/mL neomycin. Surviving populations were then sorted by flow cytometry based on iRFP expression, followed by single-cell

cloning. Candidate clones were expanded and validated by genotyping to confirm in-frame insertion of the mScarlet coding sequence at the *Cdh2* locus.

For the deletion of extracellular domain 4 (ECD4; amino acids 515–604) of *Cdh2*, *pHes7-Achilles* mESCs were transfected with sgRNA-expressing plasmids in combination with a synthetic repair double-stranded DNA fragment containing the designed sequence. Successfully transfected cells were enriched by GFP-based sorting, and individual clones were established by single-cell cloning. Genotyping confirmed the intended deletion in selected clones.

2-3 Differentiation of mESCs into iPSM

To model somitogenesis in vitro, mouse embryonic stem cells (mESCs) were differentiated into presomitic mesoderm like organoids (iPSM) following previously established protocols with modifications (Matsumiya et al., 2018; Isomura et al., 2025) (Fig.6C). This system enables controlled induction of oscillatory gene expression and permits live imaging and genetic manipulation that are not easily achieved in embryos.

2-3-1 Induction of Mesodermal Aggregates

After routine maintenance under a2i conditions, cells were dissociated and resuspended in NDiff227 medium (Takara Bio) supplemented with 20 ng/mL BMP4 (R&D Systems, 314-BP-010/CF). Approximately 2.4×10^4 cells were seeded into each well of an EZSPHERE 96-well plate (IWAKI, 4860-900), which contains multiple (~80) microwells designed to promote uniform aggregation (Sato et al., 2016). Under these conditions, each well yielded ~80 spheroidal aggregates. Cultures were maintained for 48 hours to allow mesodermal commitment.

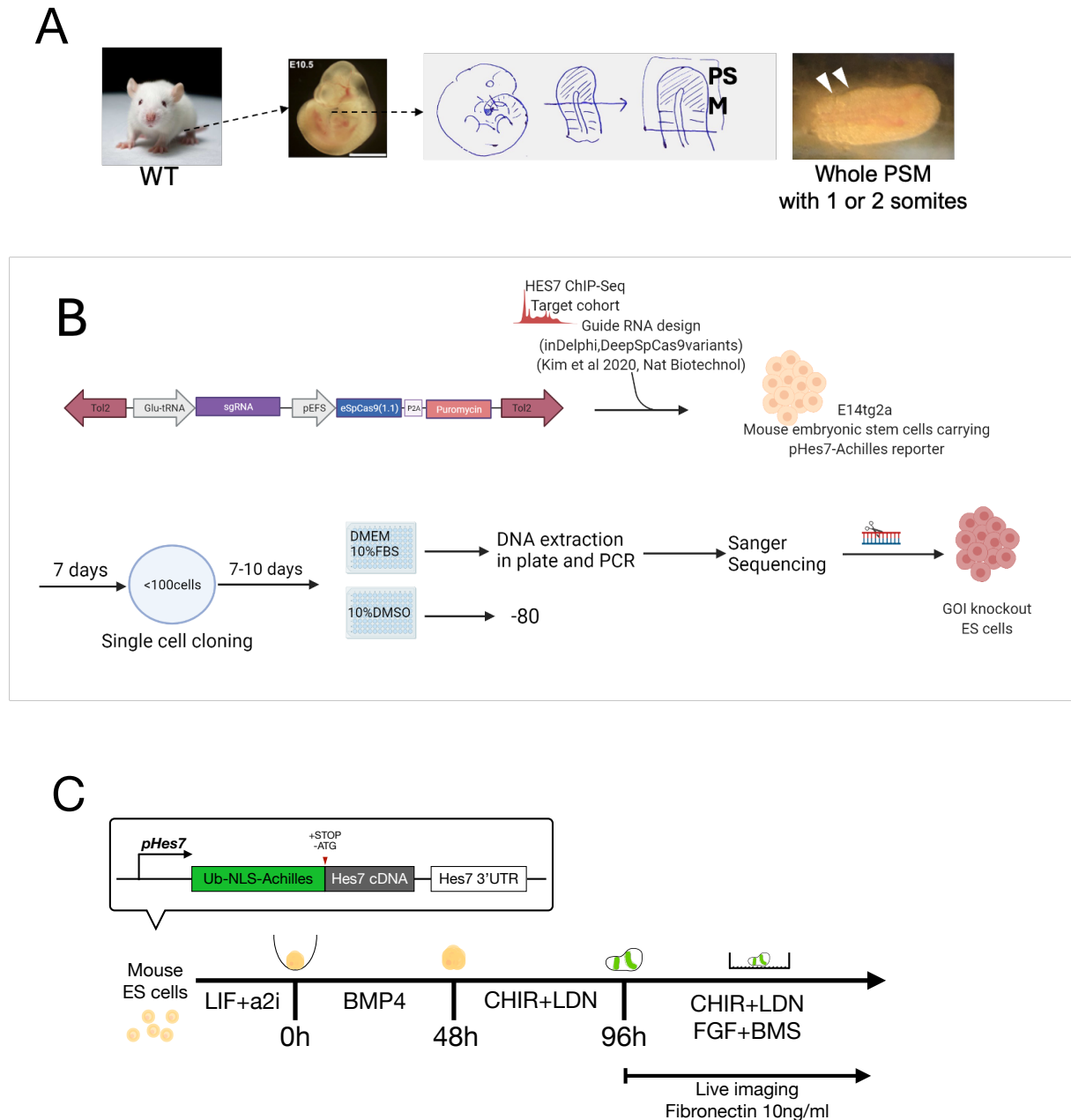


Figure 6. Schemes of experimental design. (A) Illustration of sampling for ChIP-seq on mouse PSM. (B) Illustration of the pipeline for genomic editing on genes of interest by CRISPR/cas9 and single cell line establishment. (C) The horizontal line illustrates the induction timeline. The medium should be refreshed and replaced at each indicated time point with the specific combination of reagents. After 96 hours of induction, iPSMs were plated on the fibronectin-coated plates and subjected to live imaging.

2-3-2 Transition to Presomitic Mesoderm Fate

At 48 hours, cell aggregates were washed in a wash medium consisting of DMEM/F12 (Nacalai Tesque, 05177-15) supplemented with 0.1% bovine serum albumin (BSA; Thermo Fisher Scientific, 15260-037). Aggregates were then transferred into CL medium and cultured for an additional 48 hours. The CL medium was prepared from DMEM (Nacalai Tesque, 08489-45) supplemented with GlutaMAX (Thermo Fisher Scientific, 35050-061), 1 mM sodium pyruvate (Thermo Fisher Scientific, 11360-070), 1 mM non-essential amino acids (Thermo Fisher Scientific, 11140-050), B-27 supplement lacking vitamin A (Thermo Fisher Scientific, 12587-010), 100 U/mL penicillin, and 100 µg/mL streptomycin (Nacalai Tesque, 09367-34). 1 µM CHIR99021, a Wnt pathway activator, and 100 nM LDN193189 (Sigma, SML0559), a BMP receptor inhibitor, were added to direct PSM fate. The medium contained 0.75% dimethyl sulfoxide (DMSO; Sigma, 472301-100ML) to enhance differentiation efficiency. Aggregates at this stage displayed clear presomitic mesoderm-like properties.

2-3-3 Conditions for Downstream Assays

For assays such as immunostaining and hybridization chain reaction (HCR), iPSM aggregates were maintained in CL medium until the designated fixation time points. For live imaging of oscillatory gene expression, aggregates were transferred at 96 hours into CLBF medium, which included additional signaling modulators to prolong oscillations: 2.5 µM BMS493 (Sigma, B6688-5MG), an inhibitor of retinoic acid (RA) signaling, and 50 ng/mL human recombinant bFGF (Thermo Fisher Scientific, PHG0369). These supplements preserved a posterior-like identity and stabilized *Hes7* reporter oscillations over extended culture periods.

2-3-4 Embedding for Somite Formation

To assess somite morphogenesis, iPSM aggregates were embedded in a matrix consisting of 10% Matrigel (Corning, 356231) in NDiff227 medium. Embedded aggregates were plated onto Lipidure-coated dishes (Amsbio, CM5206), which can prevent undesired adhesion. Cultures were maintained undisturbed in a humidified incubator until fixation, allowing the formation of somite-like structures.

2-4 Live imaging of oscillatory dynamics

2-4-1 Sample Preparation

To visualize dynamic changes in *pHes7* reporter activity, iPSM aggregates were transferred into CLBF medium and evenly distributed into μ -Dish 35-mm Quad chambers (ibidi, 80416). Prior to seeding, dishes were coated with 10 μ g/mL fibronectin (Corning, 354008) for at least one hour at 37 °C and rinsed with PBS. After plating, samples were incubated for 3 hours under standard conditions (37 °C, 5% CO₂) to ensure stable attachment before imaging. To minimize technical variability, control and experimental samples were always processed in parallel. For KO screening, three independent mutant lines were induced and imaged alongside wild-type controls. For inducible overexpression studies, iPSMs at 96 h were divided into two groups, with doxycycline and DMSO added to each group and set immediately prior to imaging.

2-4-2 Imaging of Dissociated Single Cells

For single-cell analysis, iPSM aggregates were collected at the 96 h and incubated with Accutase for 7 minutes at 37 °C. Gentle pipetting was performed to obtain single cells, which were subsequently pelleted by centrifugation (1800rpm for 5min) and resuspended in fresh CLBF medium. Low-density cultures were seeded into

μ -Slide 18-well glass-bottom chambers (ibidi, 81817) coated with 5 μ g/mL fibronectin, allowing individual cells to spread sufficiently for high-resolution imaging. Chambers were immediately transferred to the microscope stage for time-lapse acquisition.

2-4-3 Microscopy Setup and Acquisition

Live imaging was carried out using a Zeiss LSM980 inverted confocal microscope equipped with an environmental chamber maintaining constant temperature (37 °C), humidity, and 5% CO₂. The fluorescent Achilles reporter was excited with a 514-nm argon laser and images were collected every three minutes using a 20 $\times$ dry objective lens. For each experiment, multiple positions for each condition were defined and monitored across the imaging period to capture dynamics. All experiments were repeated at least three times for each ES cell line to ensure reproducibility.

2-4-4 Data Analysis

Fluorescence intensity data were extracted and processed using Fiji/ImageJ. The signals from the selected ROIs were plotted and analysed to visualise the oscillatory dynamics. For single-cell oscillation analysis, cells were tracked manually in bright-field mode using the TrackMate plugin (Tinevez et al., 2017). Oscillation phase was calculated by applying a Hilbert transform, and period lengths were estimated by measuring intervals between successive peaks. These approaches enabled quantitative assessment of oscillation properties at both tissue and single-cell levels.

2-5 RNA analysis

2-5-1 Quantitative PCR (qPCR)

To assess transcriptional changes of selected genes, total RNA was extracted from iPSM tissues harvested at designated time points using the RNeasy Plus Mini Kit (Qiagen). RNA integrity was confirmed prior to downstream processing, and complementary DNA (cDNA) was synthesized for quantitative PCR. Gene expression was analyzed on a QuantStudio 12K Flex system (Applied Biosystems). Transcript levels were normalized against internal housekeeping controls, β -actin and Gapdh, to correct for variability in RNA input. Primer sets were purchased from Eurofins and validated for efficiency before use in experiments.

1	qPCR primer1 <i>Cdh2</i> Fwd	TGACTGAGGAGCCTATGAAG
2	qPCR primer1 <i>Cdh2</i> Rev	TTGTAGAGCTCCACTGTGC
3	qPCR primer2 <i>Cdh2</i> Fwd	AGCCTGGAACATATGTGATGA
4	qPCR primer2 <i>Cdh2</i> Rev	CCATAAAACGTCATGGCAGTAA
5	qPCR primer <i>Hes7</i> Fwd	CGGGAGCGAGCTGAGAATAG
6	qPCR primer <i>Hes7</i> Rev	CACGGCGAACTCCAGTATCT
7	qPCR primer <i>Tbx6</i> Fwd	ATGTACCATCCACGAGAGTTGT
8	qPCR primer <i>Tbx6</i> Rev	GGTAGCGGTAACCCTCTGTC
9	qPCR primer <i>b-Actin</i> Fwd	GTGACGTTGACATCCGTAAAGA
10	qPCR primer <i>b-Actin</i> Rev	GCCGGACTCATCGTACTCC
11	qPCR primer <i>Gapdh</i> Fwd	GCCTTCTCCATGGTGGTGAA
12	qPCR primer <i>Gapdh</i> Rev	GCACAGTCAAGGCCGAGAAT

Table 2. List of primer sequences used for qPCR in this thesis.

2-5-2 Whole-Mount in Situ Hybridization Chain Reaction (HCR)

For spatial expression analysis, in situ HCR was performed on iPSM organoids and mouse embryos following adapted protocols (Alanentalo et al., 2007; Choi et al., 2018).

For iPSM organoids cultured in Matrigel, fixation was performed by adding 1 mL of 4% paraformaldehyde (PFA) in PBS directly to the Matrigel solution (300 µL per well) and incubating overnight at 4 °C. For iPSMs grown in CL or CLBF medium on fibronectin-coated dishes, the medium was removed, cells were washed gently once in PBS, and subsequently fixed in 4% PFA/PBS overnight at 4 °C. Following fixation, samples were washed three times in PBST (PBS with 0.1% Tween-20, v/v) and dehydrated in 100% methanol at –30 °C overnight for storage or further processing. Before hybridization, tissues were rehydrated by sequential PBST washes at room temperature.

For mouse embryo samples, autofluorescence from blood and yolk tissues was reduced by treating specimens in a solution of Methanol:DMSO:H₂O₂ (2:1:3; final H₂O₂ concentration 15%) at room temperature for 12–24 h after methanol dehydration. Embryos were subsequently subjected to repeated freeze–thaw cycles in 100% methanol (–80 °C to room temperature, 1 h each, repeated three to five times) to enhance probe penetration.

Whole-mount HCR v3 was carried out with reagents from Molecular Instruments. Briefly, samples were pre-incubated in 100 µL probe hybridization buffer for 30 min at 37 °C, then replaced with 1 pmol of each probe diluted in hybridization buffer and incubated overnight at 37 °C. Excess probes were removed by four washes in probe

wash buffer (15 min, 37 °C) followed by two washes in 5× SSCT buffer (10 min each, room temperature). Samples were then equilibrated in amplification buffer for 30 min.

For signal amplification, hairpins h1 and h2 were separately denatured (95 °C, 90 s), cooled to room temperature in the dark for 30 min, and then combined and diluted in amplification buffer at a final concentration of 6 pmol each in 100 µL amplification buffer. The mixture was applied to samples and incubated for 12–16 h at room temperature in the dark. Following amplification, tissues were washed twice in 5× SSCT buffer for 10min and once in DAPI-containing 5× SSCT buffer for 1 hour for nuclear counterstaining. All sticky solutions were mixed gently before incubation to ensure even reagent distribution. Finally, samples were mounted in Mount-G (Invitrogen) and sealed under a coverslip for imaging.

Probes targeting *Uncx4.1* (NM_013702.3, B1 hairpin), *Cdh2* (NM_007664.5, B1), *Mesp2* (NM_008589.2, B2), *Hes7* (NM_033041.5, B2), and *Meox1* (NM_010791.3, B1) were used in this study. Hairpin set B1 was conjugated to Alexa Fluor 647, while B2 was labeled with Alexa Fluor 594, enabling simultaneous detection of multiple transcripts.

2-6 Protein analysis

2-6-1 Western blotting

Western blot analysis was employed to evaluate protein abundance and phosphorylation states in iPSM tissues. Protein lysates were prepared and processed following established protocols (Kobayashi et al., 2019). After SDS-PAGE separation and transfer to PVDF membranes, blots were incubated with primary antibodies against the proteins of interest. The following primary antibodies were used: rabbit anti-

Actin (Sigma, A2066), rabbit anti-pFGFR1 (Cell Signaling Technology, CST 52928), rabbit anti-ERK1/2 (CST 9102), rabbit anti-phospho-ERK1/2 (CST 9101), rabbit anti-Cdh2 (CST 13116), and rabbit anti- α -Actinin (CST 6487). Secondary detection was performed using HRP-conjugated anti-rabbit IgG (GE Healthcare).

For phosphorylated proteins, optimization steps (e.g., blocking buffer composition, antibody incubation time, and washing stringency) were adjusted based on the manufacturer's technical recommendations (CST antibody datasheets). Chemiluminescent signals were acquired and quantified using actin or α -actinin as loading controls. Band intensities were normalized relative to these controls to ensure accurate comparison across samples.

2-6-2 Immunofluorescent staining

Immunofluorescence staining was carried out to examine the spatial distribution of proteins in iPSM organoids and cultured tissues, adapting procedures described previously (Veenvliet et al., 2020). Sample fixation and methanol treatment were performed following the same steps outlined in the HCR protocol, after which tissues were washed in PBST and processed for blocking and antibody incubation. To minimize reagent usage, all subsequent incubations were carried out in low-retention PCR tubes.

The following primary antibodies and working dilutions were applied: goat anti-Tbx6 (R&D Systems, AF4744; 1:200), rabbit anti-phospho-ERK1/2 (CST 9101; 1:100), rabbit anti-Cdh2 (CST 13116; 1:100), and guinea pig anti-Hes7 (home-made, Bessho et al., 2003; 1:50). Detection was achieved with species-specific secondary antibodies conjugated to Alexa Fluor dyes: Alexa Fluor 594 for Tbx6 and Hes7, and Alexa Fluor 647 for Cdh2 and ppERK.

Samples were mounted in Mount-G (Invitrogen) and sealed under glass coverslips. Imaging was performed using a Zeiss LSM980 confocal microscope. Z-stack images were acquired to capture three-dimensional structures, and projection images were generated from averaged stacks for presentation.

2-6-3 Protein Stability Assay by Cycloheximide Chase

To estimate the turnover of Cdh2 protein in iPSM cells, we employed a cycloheximide (CHX) inhibition assay. iPSM organoids were induced under standard conditions and transferred into CL medium at 96 h post-induction. Cycloheximide (Sigma, final concentration 10 $\mu\text{g/mL}$) was added to block de novo protein synthesis, and samples were harvested at multiple time points (0, 0.5, 1, 2, 3, and 4 h after treatment). Organoids were collected at each time point, lysed, and subjected to Western blot analysis using anti-Cdh2 and anti-Actin antibodies. Actin served as an internal loading control to normalize protein levels across samples. The relative abundance of Cdh2 over time provided an estimate of its half-life in PSM cells.

2-6-4 Functional perturbation by synthetic peptides

To assess whether direct physical interaction between Cdh2 and FGFR1 contributes to downstream signaling activity, a blocking peptide was synthesized through the Peptide Synthesis Service at the Support Unit for Bio-Material Analysis, RIKEN Center for Brain Science. The submitted sequence was Ac-WLKIDPVNGQI-NH₂, corresponding to the reported FGFR1-binding domain of Cdh2 (Williams et al., 2001). The peptide was delivered as lyophilized powder with >90% purity and stored at $-80\text{ }^{\circ}\text{C}$ after resuspension in DMSO at 200 mg/mL. For functional assays, aliquots were diluted into CL or CLBF culture medium at a final concentration of 200 $\mu\text{g/mL}$, vortexed, and equilibrated at $37\text{ }^{\circ}\text{C}$ for several hours before use due to its low

hydrophilicity. iPSM organoids were incubated in peptide-containing medium under humidified conditions. As a control, the same volume of DMSO was added to parallel wells. Following incubation, organoids were processed for downstream assays like immunostaining to examine the effects on ppERK signaling and PSM identity.

2-7 ChIP-seq Analysis of Hes7 Binding Sites

Presomitic mesoderm (PSM) tissue was dissected from E10.5 mouse embryos. Only the intact PSMs with 1 or 2 somites were collected to ensure tissue specificity (Fig. 6A, Fig.7). Samples were immediately transferred on ice, snap frozen in liquid nitrogen, and stored at -80°C until submitting to Active Motif custom ChIP-seq services for small-scale analysis.

Approximately 30 µg Chromatin was prepared from PSM cells. Crosslinked chromatin was fragmented and subjected to immunoprecipitation with a validated anti-Hes7 antibody (Bessho et al., 2003). Input chromatin was processed in parallel as a negative control. DNA recovered from the immunoprecipitated fraction was purified and prepared for sequencing. Then ChIP-derived DNA fragments were processed into sequencing-ready libraries using the standard Illumina ChIP-seq library construction protocol. Libraries were quality-checked prior to sequencing, and data were generated at a depth exceeding 5 million reads per sample. In total, approximately 10.5 million reads were aligned to the mouse reference genome (mm10). After computational filtering to remove duplicates and non-unique alignments, ~4.1 million high-quality reads were retained for downstream analyses.

Genome-wide signal tracks representing fragment density were generated and visualized using the Integrated Genome Browser (IGB). Peak calling was performed

using the MACS, applying a stringent threshold of $p < 1 \times 10^{-7}$ to minimize false positives. Under these conditions, 913 high-confidence Hes7 binding peaks were identified. Identified peaks were annotated with respect to genomic features such as promoters, transcription start sites, and gene bodies using the ChIPseeker R package (Yu et al., 2015; Wang et al., 2022). Visualization of peak distributions and binding profiles across loci was performed using the Integrative Genomics Viewer (IGV) online platform (<https://igv.org/app/>). These analyses enabled both the identification of known Hes7 target genes and the discovery of novel candidates relevant to somitogenesis.

2-8 Statistical analysis

All quantitative data are reported as mean $\pm$ standard error of the mean (s.e.m.), unless otherwise specified. For box-and-whisker plots, the central line represents the median, while the lower and upper hinges correspond to the first and third quartiles. Whiskers extend to the smallest and largest data points that fall within 1.5 times the interquartile range; data points outside this range were plotted individually when present. Graphical representations and statistical computations were generated in R using custom scripts.

Comparisons between two groups were evaluated using unpaired two-tailed Student's t-test, while analyses involving more than two groups were performed with analysis of variance (ANOVA), followed by appropriate post-hoc testing when required. A p-value of <0.05 was considered statistically significant. Each experiment was conducted in at least two or three independent replicates to ensure reproducibility.

Chapter 3

Result

3-1 Genome-wide identification of Hes7 direct downstream targets in the mouse presomitic mesoderm (PSM)

To investigate the regulatory function of Hes7 in the segmentation clock, I sought to perform a genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) in presomitic mesoderm (PSM) of embryonic day 10.5 (E10.5) mouse embryos by an anti-HES7 antibody (Fig.7). This homemade antibody is reported in the early studies where Hes7 is identified and examined by whole mount immunohistochemistry and ChIP analysis (Bessho et al., 2003). Here I also conducted a few experiments for validating the antibody and found the optimal conditions prior to carrying to the large-scale ChIP-seq. The immunofluorescent staining showed specific signals mapping the expected HES7 expression areas (Fig.8A), and the ChIP-qPCR confirmed the ability to specifically pull down the chromatin containing known Hes7 binding sites, such as *Lfng* and *Hes7* (Fig.8B), while no enrichment can be detected in the control serum group and non-Hes7 target gene *Math6*. These results ensured that the antibody maintained the specific recognition of Hes7 protein and was efficient for the ChIP assays. Upon confirmation, ChIP-seq on E10.5 PSM by the antibody was carried out and in total 913 binding peaks were identified corresponding to approximately 1,260 candidate target genes around these peaks (Fig. 7, 9A). These genomic sites were associated with gene promoter regions and highly biased towards the transcription start sites (TSS), indicating that Hes7 as a transcriptional repressor predominantly functions at regulatory elements of gene transcription (Fig. 9B-D). Notably, one of the strongest binding events was detected at the *Hes7* promoter itself (Table 3), consistent with the fundamental negative feedback loop where Hes7 represses its own transcription to establish oscillatory expression dynamics (Bessho

et al., 2001a, 2001b, 2003). As expected, among the targets identified, well-known mouse segmentation clock components such as *Lfng* and *Dusp4* were in the top enriched peaks confirming these genes as *Hes7* targets and also validating the specificity of the dataset. Importantly, this analysis revealed a large cohort of previously unknown candidates, including *Pcdh8* and *Cdh2*, which had been implicated in somitogenesis (Chal et al., 2017), as well as genes differentially expressed in the *Hes7* mutants, such as *Smoc1* and *Eno3* (Niwa et al., 2007). Except those mentioned above, literature research showed that the majority of the listed genes were not previously associated with somitogenesis (Jia et al., 2025). To understand the role of *Hes7* regulation in a broader view, gene ontology enrichment analysis was run on the list of identified candidates, and the most related terms were extended beyond the segmentation clock itself, including biological processes such as cell proliferation, cell death, cellular component organization, cell migration and others (Fig. 9E). Thus, I have provided a genome-wide dataset mapping the *Hes7* regulatory network in PSM and it indicated that the clock may function in a more complicated manner in regulating somitogenesis and proper timing of the biological events.

3-1-1 Selection of candidate genes for functional screening

To narrow down potential candidates for further functional analysis, firstly genes highly expressed in both mouse and human PSM were extracted by comparing the *Hes7* ChIP-seq list with the mouse and human PSM transcriptomic datasets (Diaz-Cuadros et al., 2020; Matsuda et al., 2020). The potential oscillators were also selected by referring to the gene expressed in-phase or out-phase of *Hes7* oscillations. We also included overlapping genes with more than a 2-fold change in expression in *Hes7*-null

PSM compared to *Hes7*-overexpressing PSM tissues (Niwa et al., 2007). From this list of candidates, finally I focused on a small group of 20 targets from the top for the further experiments (Table 3).

To test whether these genes contribute to the regulation of segmentation clock and oscillatory gene expression, we sought to monitor the *Hes7* oscillations to detect any defects by using a *Hes7* promoter reporter mESC line (Isomura et al, 2025) combined with gene loss of function. For each gene, two or three KO cell lines containing frameshift mutations induced by CRISPR/Cas9 were established and validated by Sanger sequencing (Fig. 10). These mESCs were differentiated into induced PSM (iPSM) tissues following a sequential signaling modulation as described in the Material and methods section where for the first 48 hours, BMP signaling was activated by adding BMP4 to the culture for guiding the pluripotent embryonic stem cells into mesoderm lineage. In the following 48 hours, the cell aggregates were transferred to a mixture condition with Wnt activation and BMP inhibition to further lead the cells to the PSM-like fate. By the 96 hours since the start of the induction, the *pHes7*-Achilles fluorescent reporter can be detected in the majority of the cells in the aggregate, indicating successful PSM differentiation. Except the KOs of *Kpnb1* and *Smc6*, the rest of KO mESCs are all able to differentiate into iPSM tissues with reporter activity (Table 3). To assess oscillatory dynamics, the iPSM tissues were imaged in a condition where FGF supplementation and RA inhibition were topped up to maintain the cells in a more posterior-like state aiming to detect any transient noises. Thus, by comparing the *Hes7* dynamics, I aimed to understand if any of the selected *Hes7* targets is indispensable in keeping robust oscillatory gene expression.

3-1-2 Screening results and identification of *Cdh2* as an important regulator of *Hes7* oscillations

To examine the *Hes7* oscillations in the KO tissues, I conducted every batch of experiments by including the wild-type cell line from the start of the iPSM induction to exclude any variables introduced by batch differences and technical inconsistency. In the wild-type iPSM tissue, *Hes7* reporter expression oscillated robustly and Achilles fluorescent waves propagated from posterior to anterior along the axis. As positive controls, *Hes7* KOs and *Lfng* KOs were first tested to check if the system can successfully recapitulate the phenotypes reported in embryos (Bessho et al., 2001; Yoshioka-Kobayashi et al., 2020). Indeed, in absence of *Hes7* itself, its promoters were kept activated leading to sustained reporter expression (Fig. 11A). In the case of *Lfng* KO, consistent with previous studies, few oscillations were detectable in the start of imaging, while quickly the travelling waves were disrupted due to desynchronization between the neighboring cells (Fig. 11C). These results suggest that this in vitro system is sensitive and efficient enough for investigating the *Hes7* dynamics in the context of segmentation clock.

Examinations of the mutant iPSMs turned out only few of the candidates exhibited phenotypes, such as *Cdh2* and *Tra2b* (Fig. 11B,D). Most of the KO tissues displayed oscillatory behaviors similar to wild-type controls (Fig. 12). In the mutant iPSMs of gene *Tra2b*, which encodes a splicing factor Vertebrate Transformer-2b (Segade et al., 1996), the embryonic bodies can be successfully induced into PSM-like cells and expressed *pHes7*-Achilles reporter, however high levels of apoptosis happened during imaging process leading to dampening of the signals eventually. *Tra2b* is essential for vertebrate development and embryos are lethal by E7.5 if *Tra2b*

is mutated on double alleles, though the detailed mechanism is still not fully understood. Few studies have indicated that it is involved in the alternative RNA splicing and its expression levels are calibrated and responsible for other genes functioning properly (Pihlajamäki et al., 2011; Roberts et al., 2014). *Xeopus* embryos with *Tra2b* knockdown showed intron retention and exon skipping of *Wnt11b* and abnormal somite patterns were observed (Dichmann et al., 2015). This gives hints that the iPSM lacking *Tra2b* might also impair the Wnt signaling which is one of the core pathways involved in presomitic mesoderm cell fate specification and differentiation, which leads to the cell death upon PSM induction. However, we decided not to focus on it since the oscillation damping obviously resulted from severe cell death and loss of reporter activity (Fig. 11D).

Instead, I also found the signals were initially normal but gradually dampened and earlier terminated in the *Cdh2* KO iPSMs while the cell viability and iPSM morphology is not much affected (Fig. 11B). In summary, among the candidates tested, *Cdh2* stood out as the only gene whose deletion specifically defected *Hes7* oscillations without triggering cell death, suggesting a further investigation into its function in segmentation clock. *Cdh2*, encoding one of the classical cadherin also called N-cadherin, is a calcium-dependent adhesion molecule with well-established roles during epithelial-to-mesenchymal transition (EMT) processes (Thevenneau and Mayor, 2012). During somitogenesis, *Cdh2* is crucial for maintaining PSM tissue integrity and coordinated cell behaviors (Duband et al., 1987; Hatta et al., 1987; Horikawa et al., 1999; Linask et al., 1998; Radice et al., 1997). Phenotypic abnormalities in somite formation and structure have been documented in *Cdh2*-deficient chicken and mouse embryos, while altered cell movements and dynamics have also been reported in

zebrafish embryos (Das et al., 2017). During somitogenesis, the initiation of first few somites formation is not affected in the *Cdh2* KO embryos while the somites and neural tubes at later stages exhibited severe abnormal structures (Radice et al., 1997). It is known that Cdh2 protein molecule as mediating cell-cell adhesion keeps the integrity of the somite structure and its internalization creates an adhesion interface allowing the future boundary characterization (Chal et al., 2017), however, its roles in the aspects of presomitic mesoderm cell differentiation and synchronized oscillatory gene expression behaviors are not illustrated at this stage. In our iPSM system, I observed that tissues with Cdh2 removed still kept morphologies comparable to wild-type ones, aside from a slight elongation (Fig. 11B), and the synchronized oscillations were detectable during the early induction period, indicating that the global cell-cell adhesion is not completely impaired. Taken together, these results suggest that Cdh2 may influence Hes7 oscillatory dynamics by causes beyond its traditional role in cell adhesion, possibly involving other mechanisms that can modulate gene expression and segmentation clock.

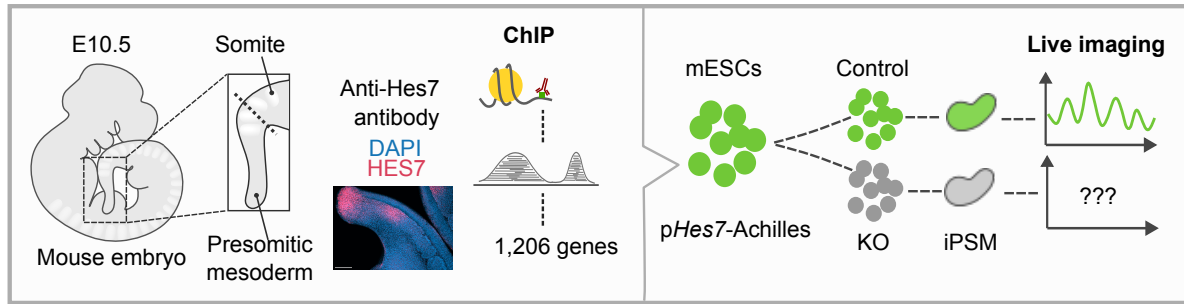


Figure 7. Experimental design of ChIP-seq and functional screening. The E10.5 mouse embryos were dissected and the caudal region containing the PSM and 2–3 newly formed somites was cut off and collected for subsequent ChIP-seq analysis. The assay used the anti-Hes7 antibody whose specificity was validated by the immunostaining confirming that it specifically binds to HES7 in the PSM (in red), and 1206 genes were identified associating with the HES7-binding peaks. A group of targets was selected for further screening and knockout lines of mouse ES cells of these genes were imaged to screen potential factor involved in regulating Hes7 oscillations.

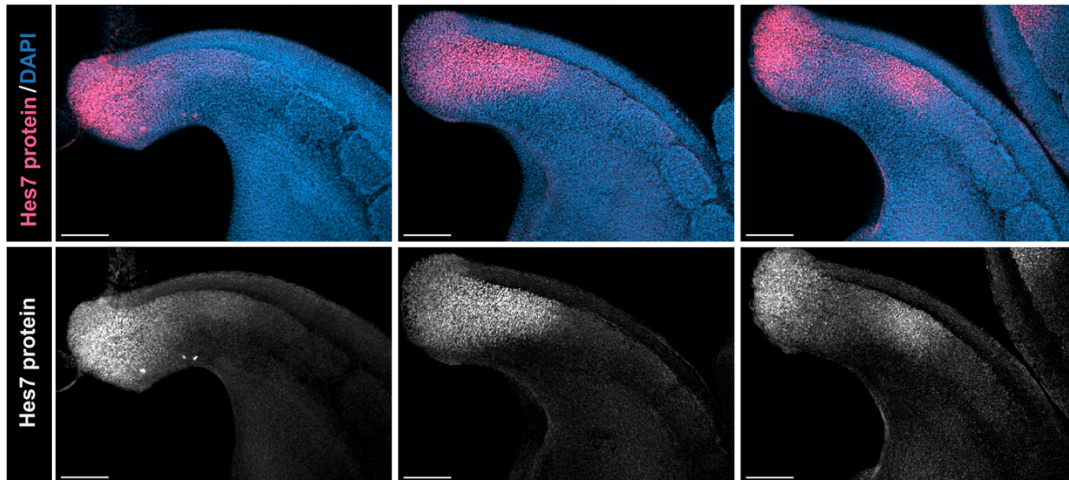
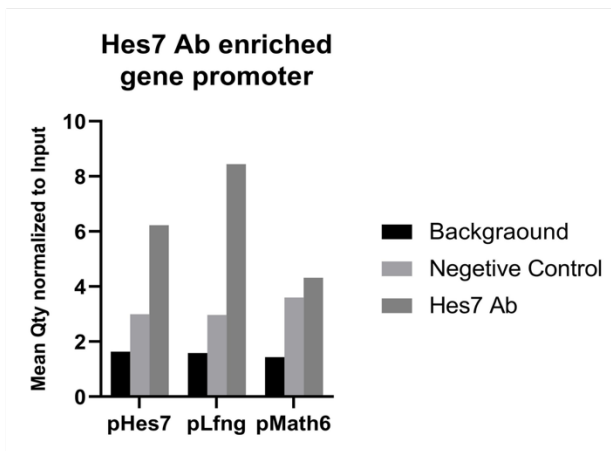
A**B**

Figure 8. Validation of anti-Hes7 antibody by whole-mount immunofluorescent staining of E9.5 embryos (A) and ChIP-qPCR (B). (A) Representative immunofluorescent images of Hes7 protein (red) expression patterns merged with DAPI (blue) in the E9.5 mouse PSM. Bottom images are the same but show Hes7 protein (red) expression patterns only. Images are the average intensity projections of 20 μm z-stacks. Scale bars, 100 μm . (B) The immunoprecipitated promoter fragments of *Hes7*, *Lfng* and *Math6* were quantified by real-time qPCR ($n = 100$ PSMs; $N = 1$ experimental repeat).

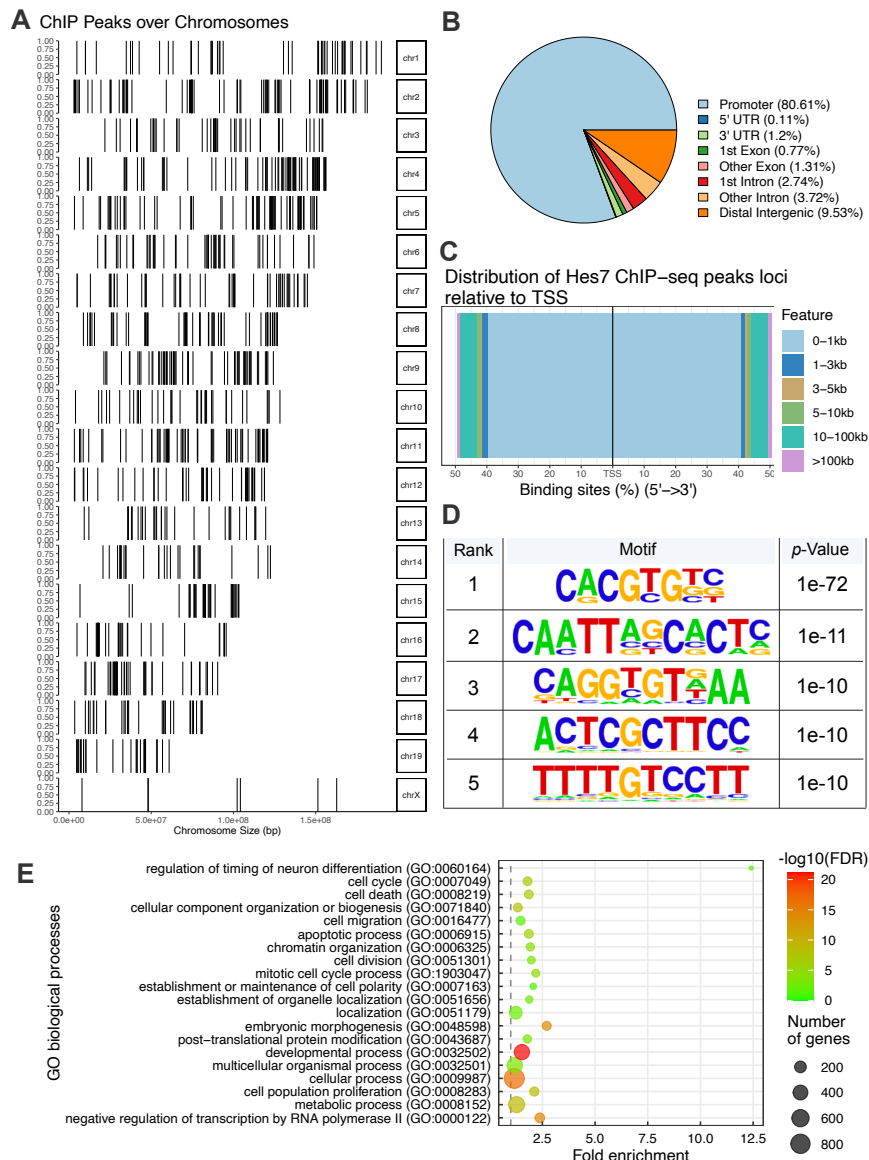


Figure 9. Identification of genome-wide Hes7 binding sites by ChIP-seq. (A) The distribution of reads on chromosomes from ChIP-seq data. (B) Pie diagram showing the genomic occupancy of Hes7 binding sites as revealed by ChIP-seq in the PSM. (C) Bar diagram showing the percentage of Hes7 binding sites upstream and downstream from the transcription start site (TSS) of the nearest gene. (D) Top five predicted Hes7 binding motifs with the most significant p-values. (E) Top 20 enriched Gene Ontology (GO) terms of the Hes7 targets.

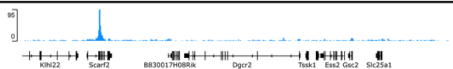
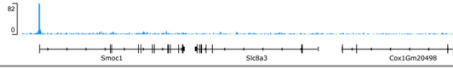
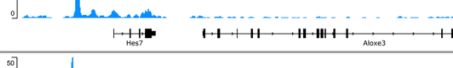
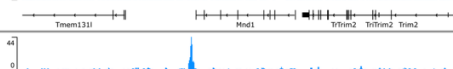
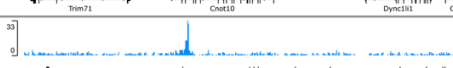
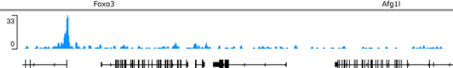
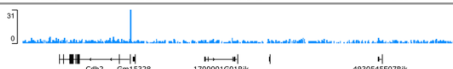
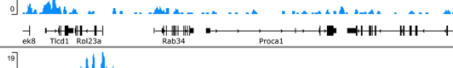
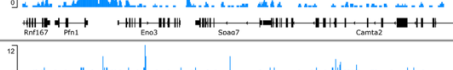
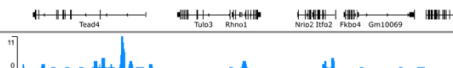
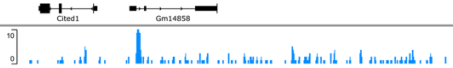
Rank	Gene	Hes7 ChIP-seq binding	Phenotype	GO term
2	Scarf2		NC	Cell-cell adhesion
4	Smoc1		NC	extracellular matrix organization
6	Lfng		Desynchro- nization	Notch signalling pathway
9	Hes7		Sustained expression	Negative regulation of transcription by RNA polymerase II
12	Irf2bp2		NC	Negative regulation of transcription by RNA polymerase II
13	Kpnb1		ND	Protein import into nucleus
17	Tmem131l		NC	Negative regulation of canonical Wnt signaling pathway
19	Khdrbs1		NC	mRNA processing
26	Trim71		NC	miRNA processing
37	Foxo3		NC	Regulation of transcription by RNA polymerase II
40	Jak3		NC	Intracellular signal transduction
46	Cdh2		Dampened	Cell migration
53	Bcl2l11		NC	Programmed cell death
61	Pcbp1		NC	Positive regulation of transcription by RNA polymerase II
79	Ier5		NC	Positive regulation of transcription by RNA polymerase II
88	Rab34		NC	Endocytosis
142	Eno3		NC	Glycolytic process
482	Tead4		NC	Hippo signaling
611	Cited		NC	Positive regulation of transcription by RNA polymerase II
639	Tra2b		Cell apoptosis	Regulation of RNA splicing

Table 3. ChIP-seq profiles for genes selected for knockout screening. The Hes7 binding peaks in mouse E10.5 PSM are visualized using the online IGV Browser. NC: no change; ND: no data.



Figure 10. Genotype sequencing of selected genes for KO screening. KO mutants generated by gene editing were sequenced.

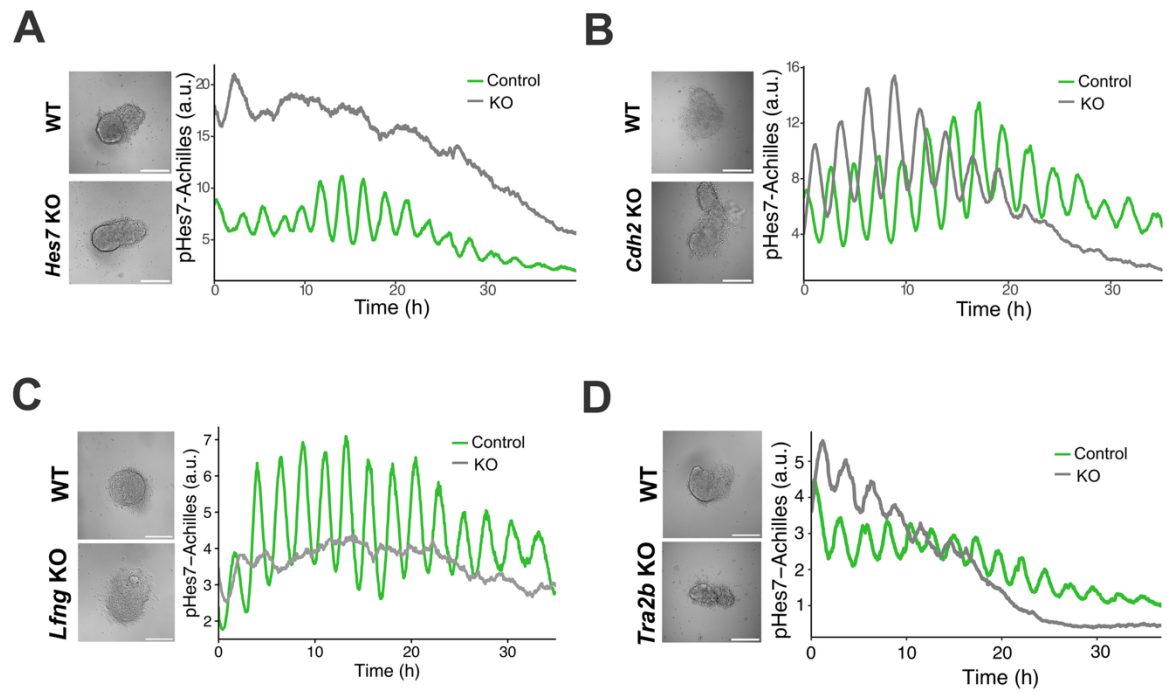


Figure 11. Time series plots of *pHes7-Achilles* activity in knockout iPSM tissues with phenotypes. *Hes7* KO (A), *Cdh2* KO (B), *Lfng* KO (C), and *Tra2b* KO (D) iPSMs were analyzed. a.u., arbitrary units.

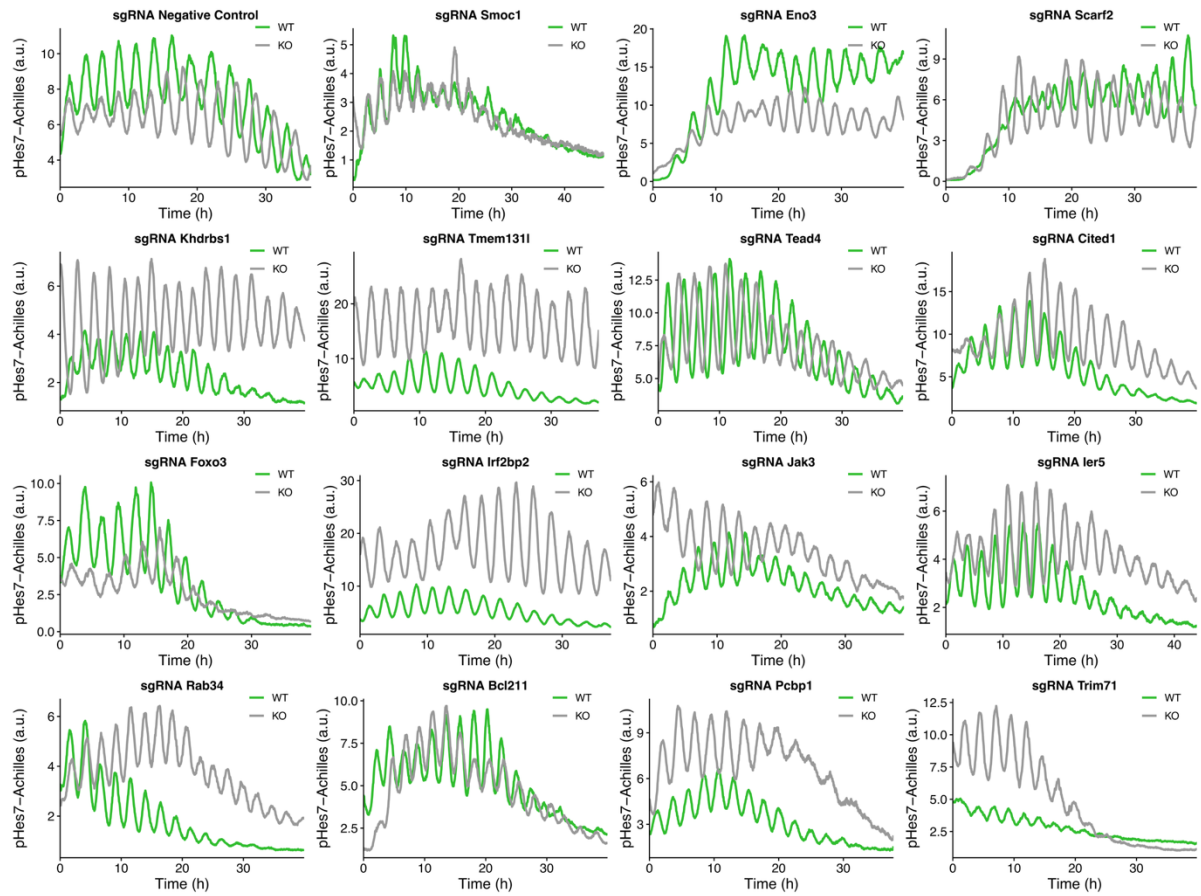


Figure 12. Time series plots of *pHes7*-Achilles activity in knockout iPSM tissues showing no phenotype. a.u., arbitrary units.

3-2 Exploring the relationship between Hes7 regulation and Cdh2 expression in mouse presomitic mesoderm

3-2-1 Hes7 represses Cdh2 transcription and protein levels

To investigate how *Cdh2* expression is regulated by Hes7 within the PSM, I firstly sought to confirm whether Hes7 binding on the *Cdh2* promoter is sufficient to suppress its transcription by examining *Cdh2* levels with exogenous high Hes7 expression. This is achieved by using an inducible Tet-ON system where Hes7 can be induced at any time points during the iPSM induction (Fig. 13A). Here, doxycycline was administered at 96 hours post-induction to trigger sustained Hes7 expression, which makes sure of Hes7 overexpression after cells had already entered a PSM fate. Quantifications of mRNA amounts after 24 hours of increased Hes7 expression (Fig. 13B) showed that *Cdh2* mRNA levels were significantly reduced (Fig. 13D), while only small changes were observed in the expression of *Tbx6*—a marker of PSM identity (Fig. 13C). This indicates that Hes7 is capable of repressing *Cdh2* transcription without broadly affecting the PSM cell properties. At the cellular protein level, iPSM tissues with elevated Hes7 exhibited cluster formation visualized by mCherry expression, where the *Cdh2* protein detected was reduced relative to the neighboring cells (Fig. 13E). These findings suggest that Hes7 upregulation promotes the local downregulation of *Cdh2* transcription subsequently reduction of *Cdh2* protein present on cell membranes, which in turn causes these cells segregate from Hes7-low/*Cdh2*-high populations, reflecting a change in adhesion-based tissue reorganization.

To validate the relationship further, I compared in situ *Cdh2* mRNA distribution in wild-type (WT) and *Hes7* KO iPSM tissues embedded in Matrigel at 120 hours post-induction. In the WT iPSMs, *Cdh2* showed a gradient with higher expression at the

posterior tip that decreased toward the anterior region reaching the lowest point where the boundary marker *Mesp2* was expressed (Fig. 14A). Then the expression was upregulated after cells committed to the somite fate. By contrast, *Hes7* KO iPSMs displayed a stable *Cdh2* mRNA level along the posterior-anterior axis until *Mesp2* strips, resulting in a relatively flattened gradient (Fig. 14B). This demonstrates that *Hes7* is required for repressing *Cdh2* transcription and regulating the *Cdh2* expression levels in PSM.

3-2-2 Dynamic *Cdh2* mRNA expression in PSM

To gain deeper insight into how *Cdh2* is related to segmentation clock, *Cdh2* mRNA distribution in mouse embryos is visualized by in situ Hybridization chain reaction (HCR). Similar to the observation of iPSM tissues, *Cdh2* mRNA is also enriched in the tailbud regions and nascent somites, while within the PSM it displayed much lower expression and wave-like fluctuations coupling the *Hes7* expression patterns (Fig. 15). However, unlike *Hes7*, whose on-off domains are sharply presented, *Cdh2* transcription is comparatively shallow and graded, with only subtle differences between high and low expressing zones. These observations in vivo suggest that while *Cdh2* transcription is periodically repressed by *Hes7*, its oscillatory dynamics are less robust and contribute primarily to a gradual reduction of expression in the anterior PSM.

3-2-3 Gradient-like distribution of *Cdh2* protein in the PSM

Next, I assessed *Cdh2* protein distribution in mouse PSM by immunostaining to understand whether the similar dynamics also exhibited in the protein level. Unlike the fluctuating mRNA patterns, *Cdh2* protein displayed a relatively stable posterior-to-

anterior gradient among the tested samples, with high levels in posterior regions, progressively reaching lowest levels in the anterior domains and increased in the newly formed somites (Fig. 16). This indicated that likely dynamics at protein level might be much weaker or any post-transcriptional mechanisms, particularly protein stability, may buffer transcriptional fluctuations.

Considering the limitation to catch subtle dynamics in fixed samples, a *Cdh2*-mScarlet fusion reporter line is generated by knocking in fluorescent protein mScarlet to the C terminal of *Cdh2* locus in the *pHes7*-Achilles reporter line (Fig. 17A). The iPSM tissues induced from these ESCs allow simultaneous monitoring of *Cdh2* protein localization and *Hes7* oscillations by live imaging. Following the same induction procedures, robust *Hes7* traveling waves via Achilles reporter activity and membrane-localized *Cdh2*-mScarlet signals were detected, confirming the correct protein visualization and successful differentiation (Fig. 17B). However, consistent with immunostaining patterns, no clear oscillatory protein waves were detected in the time-lapse imaging (Fig. 17C). Similarly, a persistent posterior-to-anterior descending gradient was observed throughout the imaging (Fig. 17D).

The discrepancy between the dynamic mRNA patterns and stable protein gradients might be explained by differences in protein turnover rates. *Hes7* protein is known to be highly unstable, with a half-life of approximately 22 minutes, a feature essential for its oscillatory behaviors (Hirata et al., 2004). So, we conducted cycloheximide chase experiments and revealed that *Cdh2* protein has a much longer half-life of about 3 hours (Fig. 18A-C). This extended stability would allow the protein amounts to maintain even as the transcriptional inputs fluctuate, resulting in smooth protein gradients despite oscillatory mRNA regulation.

Together these findings establish a model in which Hes7 periodically represses *Cdh2* transcription, generating shallow wave-like mRNA dynamics in PSM. However, because Cdh2 protein is relatively stable, its expression is translated into a stable descending gradient along the posterior-anterior axis, rather than oscillations. This gradient requires periodical repression by Hes7 and might influence cell reorganization and boundary formation during somitogenesis by differential adhesion properties as cell transition from posterior to anterior end.

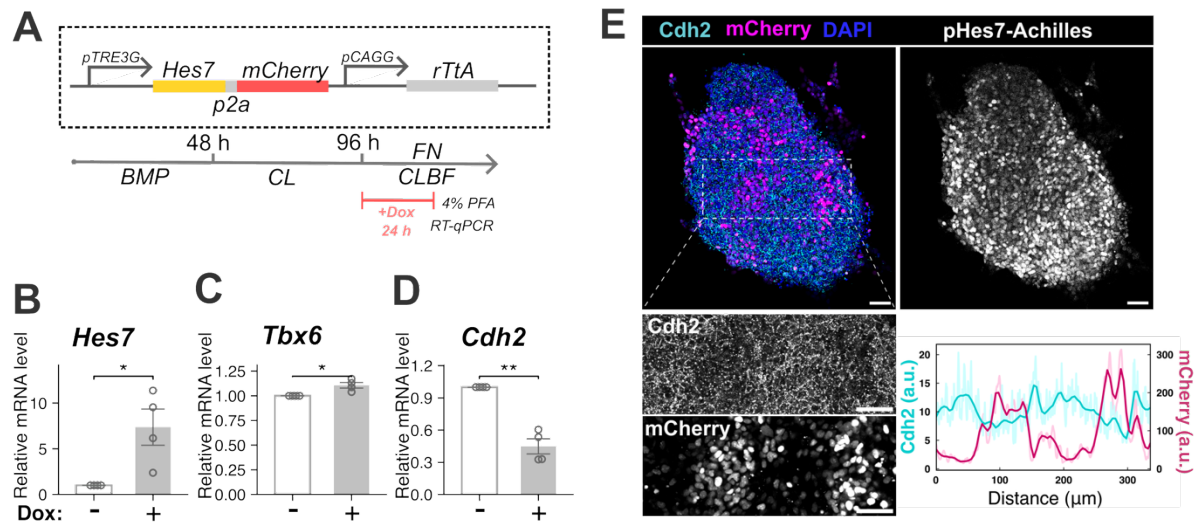


Figure 13. Hes7 represses *Cdh2* transcription and protein levels. (A) Scheme of the Hes7-overexpression vector (top) and the doxycycline administration schedule (bottom). (B-D) Expression of *Hes7* (B), *Tbx6* (C) and *Cdh2* (D) mRNA was quantified by real-time qPCR. Data are shown as the mean $\pm$ SEM, , t-test: * $p \leq 0.05$, ** $p \leq 0.01$. (E) Representative Immunofluorescence images of iPSM tissue overexpressing Hes7. The rectangular ROI is enlarged to visualize the distribution more clearly. Cdh2 protein intensity was plotted from left to right along with mCherry intensity. Scale bars, 50 μ m. a.u., arbitrary units.

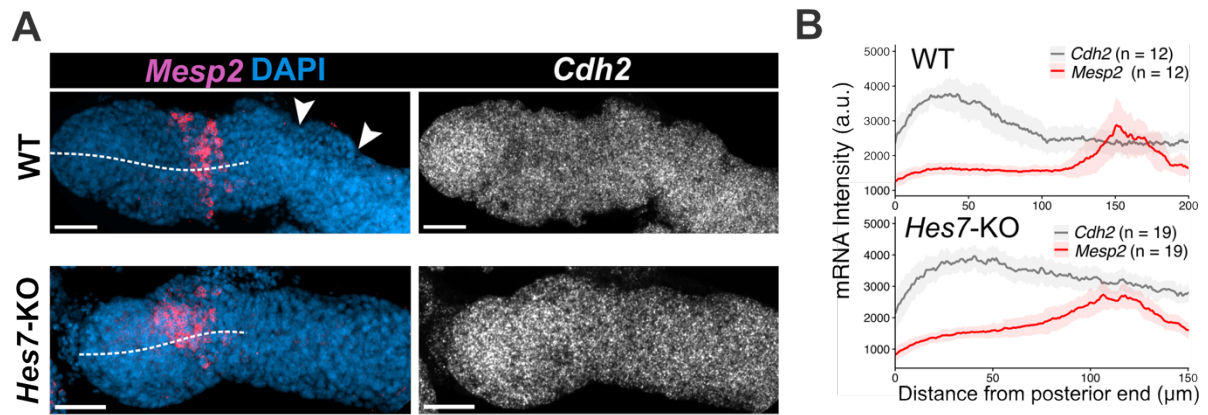


Figure 14. Hes7 is required for repression of Cdh2 expression at anterior iPSM tissue. (A) Representative hybridization chain reaction staining images of *Mesp2* mRNA expression (magenta), DAPI (blue), and *Cdh2* mRNA expression (grey). A dotted white line was drawn along the central axis of each iPSM, from the posterior end to the termination of the *Mesp2* stripe, to measure and plot intensity along the axis. Scale bars, 100 μm. Arrowheads indicate the segment boundaries. (B) Spatial plot of *Mesp2* mRNA expression along the posterior-anterior axis. Data are the mean $\pm$ 95% confidence interval, with the 2.5th and 97.5th percentiles shown for each data point. a.u., arbitrary units.

E9.5 mouse PSM

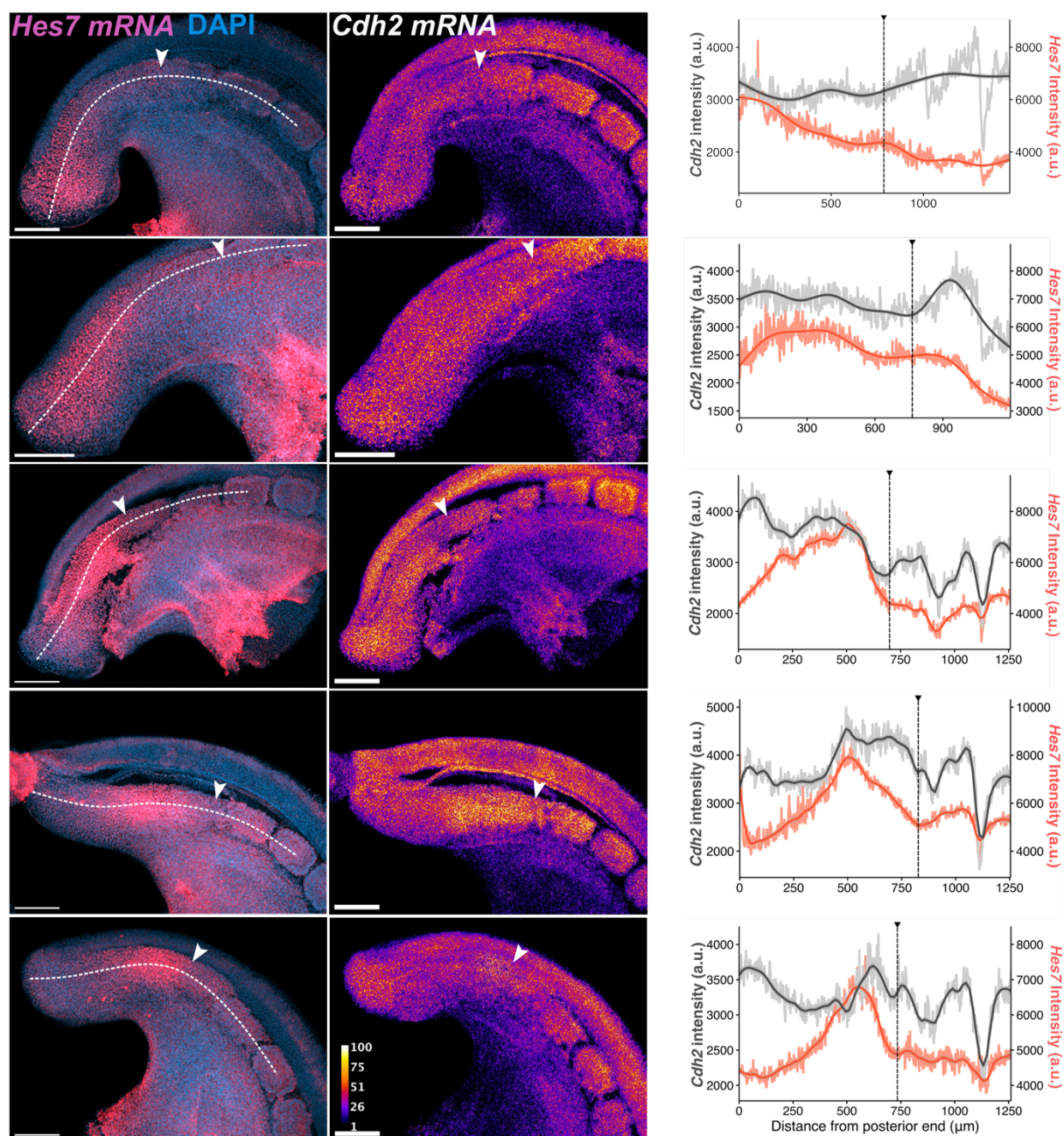


Figure 15. *Cdh2* mRNA expression in mouse E9.5 PSM. Left: Representative hybridization chain reaction staining images to examine *Cdh2* mRNA (fire) and *Hes7* mRNA (red) expression patterns in E10.5 mouse PSM. Right: Intensity profile was plotted along the indicated lines shown in the left panels. a.u., arbitrary units. The vertical lines with arrowheads indicate the boundary between the PSM and somites,

which correspond to the white arrow heads in the images. 0 indicates the posterior end. Images are the average intensity projections of 20 μm z-stacks. Scale bars, 100 μm .

E10.5 mouse PSM

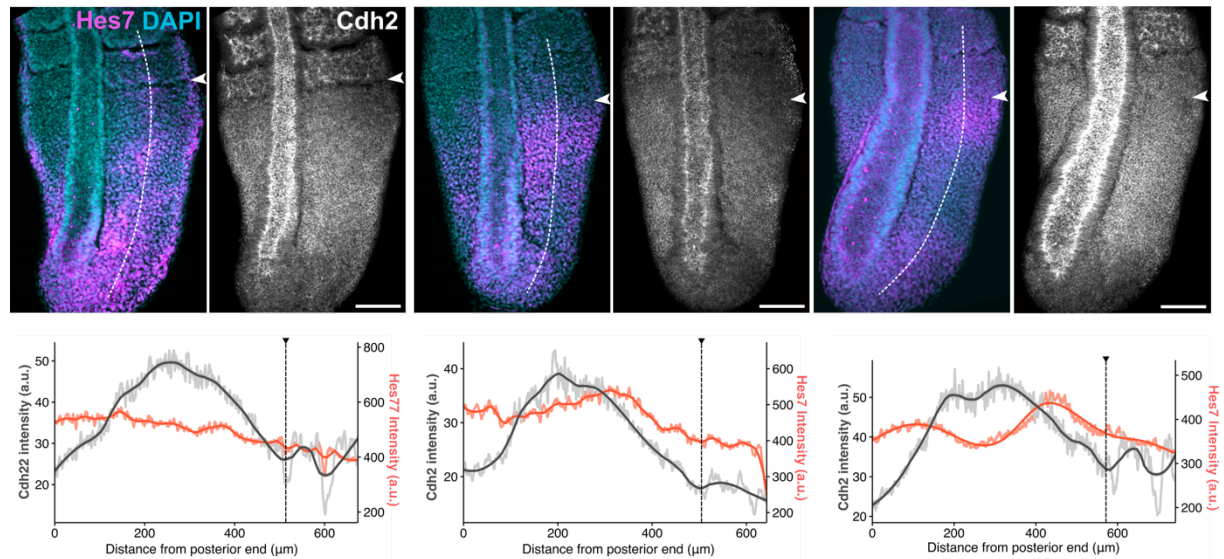


Figure 16. Cdh2 protein distribution in mouse E10.5 PSM. Top: Representative immunofluorescent images of Cdh2 protein (grey) and Hes7 protein (red) expression patterns in E10.5 mouse PSM. Bottom: Intensity was plotted along the indicated lines shown in the top left panels. a.u., arbitrary units. The vertical lines with arrowheads indicate the boundary between the PSM and somites, which correspond to the white arrow heads in the images. 0 indicates the posterior end. Images are the average intensity projections of 20 μm z-stacks. Scale bars, 100 μm .

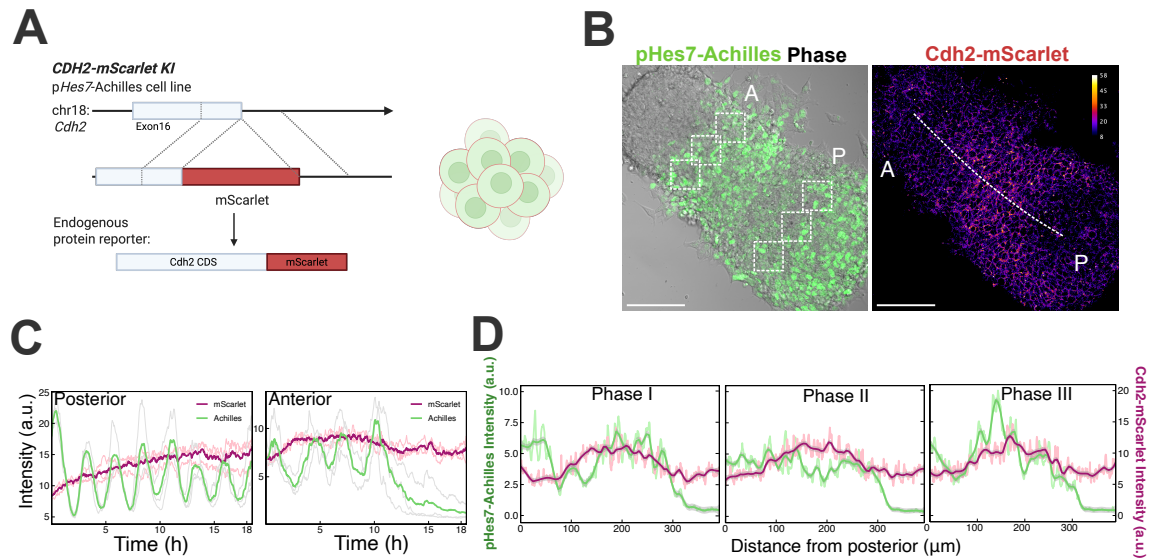


Figure 17. Cdh2 protein exhibits a relatively stable gradient along the posterior to anterior axis in iPSM. (A) Scheme of the knock-in strategy for generating a Cdh2-mScarlet fusion protein reporter line. (B) Representative snapshots of Achilles and mScarlet channels 6 h after starting live imaging (105-h post induction). Dashed squares indicate the ROIs for quantitative plot of intensity. P: posterior; A: anterior. Dashed line in the mScarlet channel is used for plotting intensity along the P-A axis. Scale bars, 100 μ m. (C) Plots of the pHes7-Achilles intensity along time measured by using the ROIs in (B). The purple and green lines indicate the mean of intensities of Cdh2-mScarlet and pHes7-Achilles, respectively, from 3 ROIs, and the light pink and grey lines represent the intensity from each single ROI. (D) Spatial smoothed plots of intensity from pHes7-Achilles (green) and CDH2-mScarlet (purple) channels along the posterior-anterior axis. a.u., arbitrary units.

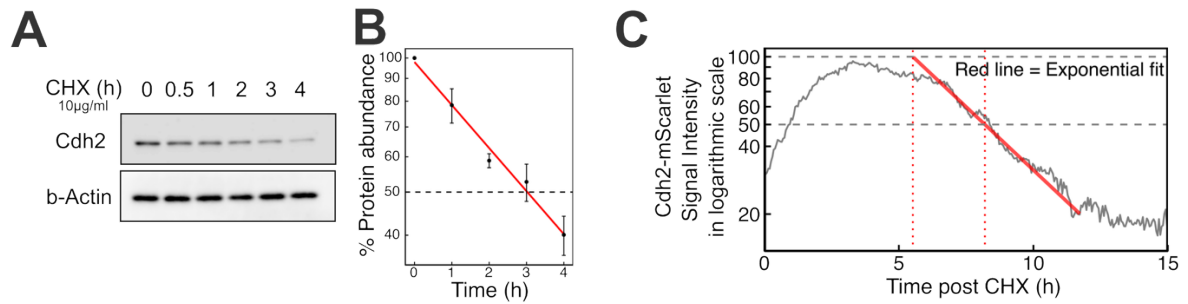


Figure 18. Cdh2 protein has a half-life about 3 hours. (A) CDH2 protein levels were quantified by western blotting of wild-type iPSM collected from a time series post CHX administration. (B) Quantification of Cdh2 protein abundances shown as means $\pm$ SEM for each time point and are fitted to the logarithmic scale presented as the red line. (C) The Cdh2-mScarlet signal intensity in logarithmic scale measured by live imaging with CHX administration. The grey line is the mean value, and the red line is the exponential fitting to the slope of the mean intensity. Dashed grey lines denote the values ($y=100$, $y=50$) and corresponding time ($x=5.52$, $x=8.2$) is denoted by red dotted lines.

3-3 Cdh2 expression levels influence Hes7 oscillations and timing for differentiation

To understand why removing Cdh2 leads to early termination of Hes7 oscillations and how Cdh2 levels would impact segmentation clock, I analyzed Hes7 oscillations in iPSM systems with high Cdh2 expression exogenously induced. An overexpression cell line was established by introducing a Tet-ON construct carrying Cdh2 coding sequence with a p2a-mCherry (Fig. 19A). This allows Cdh2 overexpression at any favorable timing by simply adding doxycycline into the culture medium and the intensity of exogenous Cdh2 protein can be indicated by the p2a-mCherry signal intensity. Successful upregulation of global Cdh2 protein levels was confirmed by western blotting following doxycycline treatment (Fig. 22C). In parallel, a control cell line is generated similarly but expressing only mCherry without the Cdh2 coding sequence (Fig. 20A). These control tissues displayed normal morphology and elongation in both treated and untreated groups comparable to the wild-type iPSMs (Fig. 20B). By contrast, upon induction of Cdh2 overexpression, iPSM tissues lost their clear posterior-anterior axis, and cells became more dispersed relative to the untreated samples (Fig. 19B-C). These morphological changes suggest that elevated Cdh2 expression modifies tissue organization and possibly disrupts directional elongation.

To characterize the Hes7 oscillatory behaviors with high Cdh2 levels, I monitored the p*Hes7*-Achilles reporter activity in the presence of doxycycline treatment. In both control and Cdh2-overexpressing iPSM tissues, administration and the subsequent exogenous protein load weakened overall *Hes7* reporter signals (Fig. 20G), possibly due to the extra consumption of energy by the overexpressing system. However, differences in oscillatory dynamics were evident between groups. In control

tissues with only mCherry expressed, Hes7 oscillations gradually diminished in the anterior region after a few waves, consistent with the normal process observed in wild-type where PSM cells downregulate Hes7 as they transition toward differentiation (Fig. 20C-E). By contrast, iPSM tissues overexpressing *Cdh2*, Hes7 oscillations persisted for a markedly longer duration, with sustained *pHes7*-Achilles activity detectable especially around anterior regions until the end of live imaging (Fig. 19C-D, 20F). This indicates that high levels of *Cdh2* prolong the oscillatory state and delay the silencing of Hes7 expression that usually accompanies PSM differentiation.

To further assess whether the altered duration of Hes7 expression is a result of changed PSM identity, I examined the expression pattern of *Tbx6*, a key transcription factor that marks the PSM character and can be used to distinguish the PSM-like cells from the differentiated parts. Indeed, in *Cdh2* high tissues, there is an increased percentage of *Tbx6*-expressing cells compared with wild-type controls (Fig. 21A-C), suggesting prolonged maintenance of PSM-like identity. Conversely, *Cdh2* KOs showed a reduction in *Tbx6*-positive proportion (Fig. 21A-C) with increased differentiated cells. These results suggest that *Cdh2* levels can directly influence the timing whether PSM cells maintain an undifferentiated state or proceed toward somite fates.

Because Notch signaling is a major pathway that coordinates segmentation clock and acts directly upstream of Hes7, I next investigated whether *Cdh2* regulates Hes7 dynamics via mediating Notch activity. Surprisingly, levels of Notch intracellular domain (NICD) were not significantly changed in the *Cdh2* KO iPSM tissues (Fig. 22A-B), indicating that the removal of *Cdh2* does not disrupt basic Notch signaling or cell-cell contact-dependent Notch activation. In contrast, upregulation of *Cdh2* in iPSM cells

leads to decrease of both NICD (Fig. 22C-D) and Lfng (Fig. 22C, E) protein levels, weakening the Notch activity, which could not explain the prolonged oscillations observed with Cdh2 overexpression. Therefore, other mechanisms or signaling pathways may be involved in these phenotypes.

These experiments together demonstrate that Cdh2 expression levels are crucial for controlling Hes7 oscillatory activity and the timing of exiting this state. High levels of Cdh2 prolong Hes7 oscillations and delay this exit as reflected in extended Tbx6 expression. Importantly, this regulatory effect is not mediated by changes in Notch signaling, suggesting that Cdh2 may act through alternative pathways.

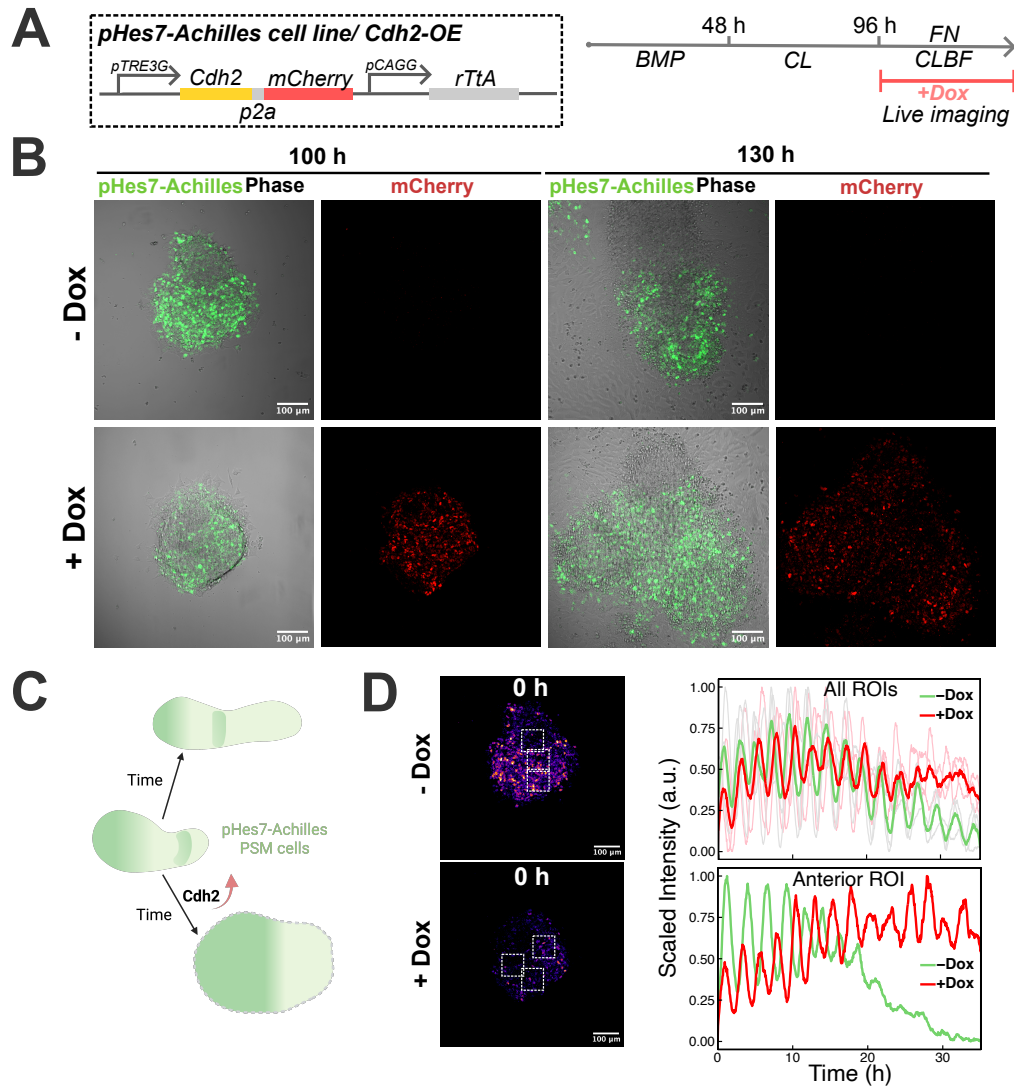


Figure 19. Delayed differentiation of iPSM tissue by Cdh2 overexpression. (A) Scheme of the overexpression (OE) vectors (left) and iPSC induction protocol (right). (B) Representative snapshots of Achilles and mCherry channels at 100 h and 130 h. (C) Scheme of summarizing the phenotype of Cdh2-OE iPSC. (D) Representative snapshots of *pHes7-Achilles* channels in Fire at 0 h after starting imaging (10-h post induction) (left) and the plot of *pHes7-Achilles* intensity along time measured by using ROIs in the left panel (right top) and a single anterior ROI (bottom). The purple and green lines indicate the mean of intensities from 3 ROIs, and the light pink and grey lines represent the intensity from each single ROI.

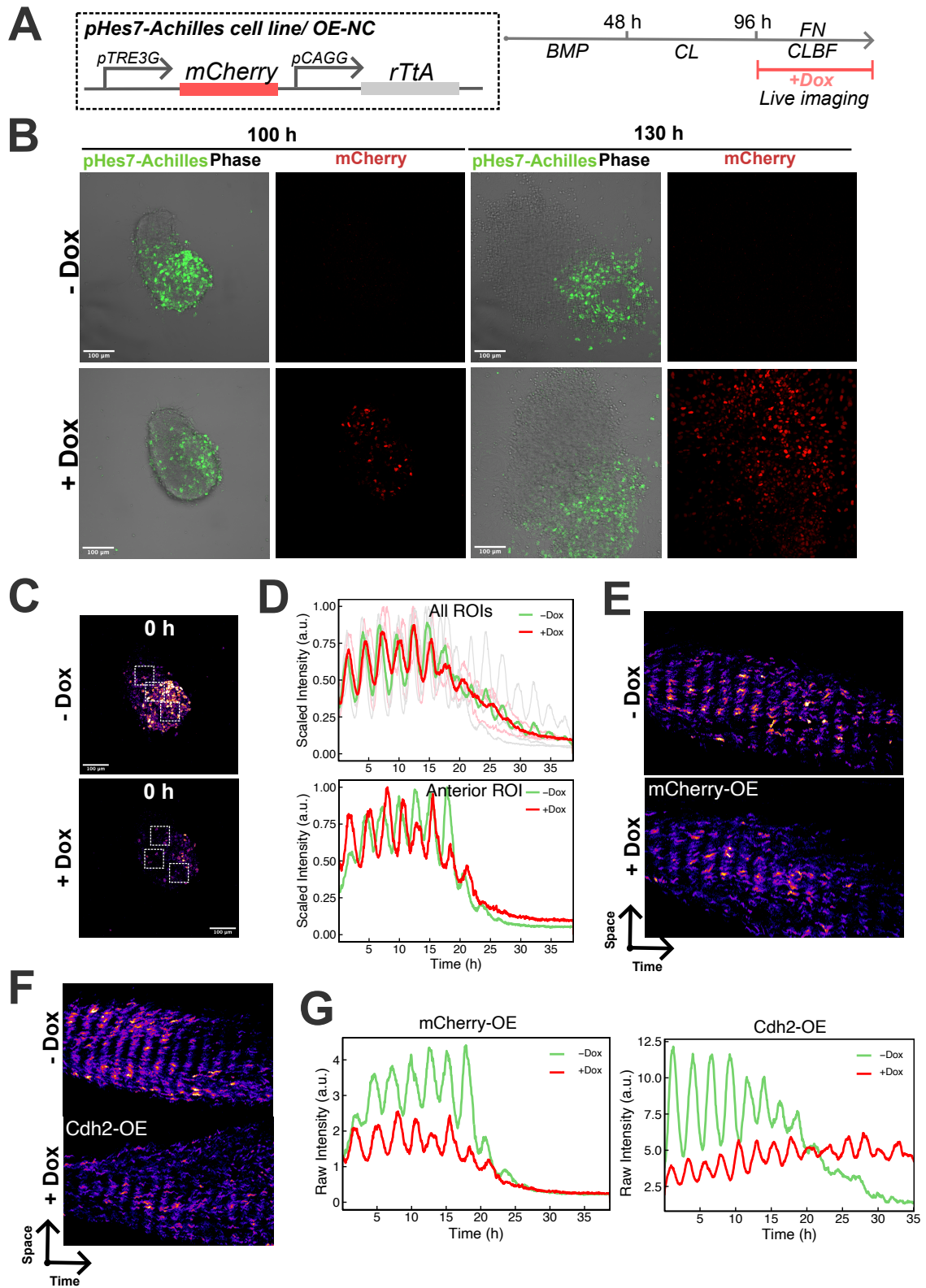


Figure 20. Overexpression of mCherry in iPSC tissues did not affect Hes7 oscillations. (A) Scheme of the overexpression construct (left) and iPSC induction

protocol (right) (B) Representative snapshots of Achilles and mCherry channels at 100 h and 130 h. (C) Representative snapshots of *pHes7*-Achilles channels in Fire at the start of imaging (130-h post induction). (D) Plots of scaled *pHes7*-Achilles intensity along time measured in ROIs indicated in (C). All ROIs (top) and single anterior ROI (bottom). The thick red and green lines indicate the mean of intensities from 3 ROIs, and the light pink and grey lines represent the intensity from each single ROI. (E-F) Kymographs of spatial and temporal *pHes7*-Achilles signals with mCherry-OE (E) and Cdh2-OE iPSM (F). (G) Plots of raw *pHes7*-Achilles intensity for both –Dox (control) and +Dox (mCherry overexpression in mCherry-OE and Cdh2-p2a-mCherry overexpression in Cdh2-OE). a.u., arbitrary units.

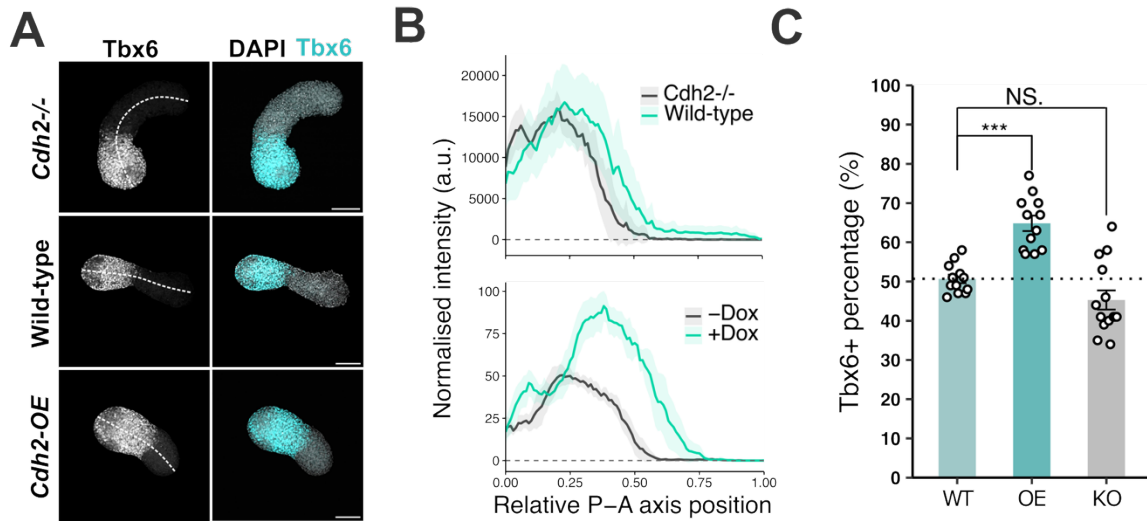


Figure 21. Proportions of PSM-like cells (Tbx6+) in iPSM tissues are related to the *Cdh2* levels. (A, B) Representative images of Immunofluorescent staining (A) of iPSM tissue in CL medium at 108 h and corresponding signal measurement (B) along the posterior-anterior axis of Tbx6, where 0 is the posterior end and 1 is the anterior end (n = 12 iPSM tissues for each condition). Data are the mean $\pm$ 95% confidence interval, with the 2.5th and 97.5th percentiles shown for each data point. a.u., arbitrary units. (C) Bar chart comparing the Tbx6-positive percentage for each condition. The values are extracted from the plot (B), data are shown as mean $\pm$ SEM. T-test, *** $p \leq 0.001$, NS: not significant.

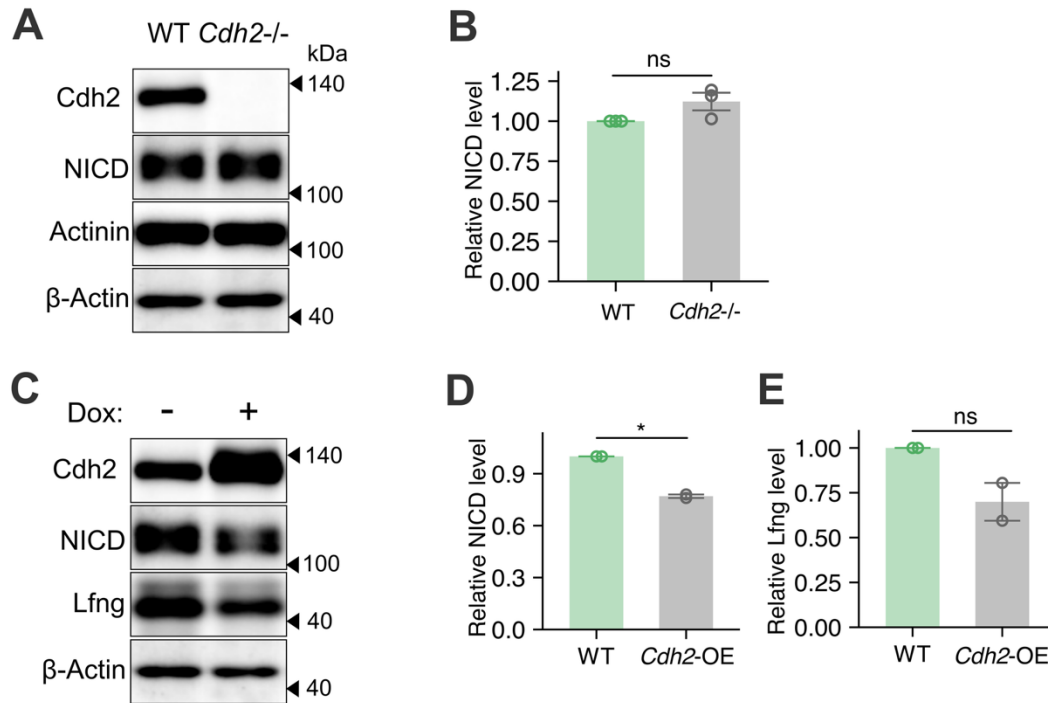


Figure 22. Effect of Cdh2 on Notch signalling activity in iPSM tissue. (A, B) NICD levels were quantified by western blotting. WT and *Cdh2*^{-/-} iPSM samples were collected at 96 h. (C-E) NICD and Lfng protein levels were quantified by western blotting. Control (Dox-) and Cdh2-overexpressing (OE, Dox+) iPSM tissues were collected at 108 h. Doxycycline was administered at 96 h. Data are shown as the mean $\pm$ SEM, t-test: * $p \leq 0.05$, ns: not significant.

3-4 Cdh2 levels modulate FGF/ppERK signaling activity in presomitic mesoderm cells

In vertebrate somitogenesis, the posterior-to-anterior gradient of FGF signaling is a key regulator of segment boundary positioning (Anderson et al., 2020; Dubrulle et al., 2001; Dubrulle and Pourquié, 2004; Niwa et al., 2007; Sawada et al., 2001; Wahl et al., 2007). The oscillatory activity of its downstream effector ppERK coordinates with the segmentation clock to ensure timely somite formation (Niwa et al., 2011; Simsek et al., 2023). Importantly, inhibition of FGF signaling has been shown to completely abolish Hes7 expression in the presomitic mesoderm (Niwa et al., 2007) even with Notch signaling still presented. This highlights that Hes7 expression and oscillations depend on both Notch and FGF signaling while the initiation is highly rely on FGF signaling, and that Notch signaling is more responsive for propagation of oscillations. Furthermore, previous studies have reported the association of Cdh2 and FGF signaling activity in other cell types including tumor cells (Suyama et al., 2002), neurons (Kon et al., 2019; Williams et al., 2001) and epiblast stem cells (Takehara et al., 2015), where Cdh2 can stabilize FGFR1 on the membrane through direct physical interactions preventing internalization and maintaining FGF/ppERK activity (Fig. 23A). However, whether the regulatory relationship is also present in PSM remains unclear. Since the Notch signaling is not much affected when Cdh2 is removed from PSM cells, it is likely that Fgf signaling was causing the inactivation of Hes7 expression and oscillations.

To test whether the early termination of Hes7 oscillations in *Cdh2* KO tissues was linked to impaired FGF signaling activity, the ppERK levels were measured as an indicator of downstream FGF signaling activation. The total protein lysis was extracted

and prepared from 96-hour iPSM cultures, where the majority of the cells in the tissues still exhibited posterior PSM-like identity at this time point (Fig. 23A). Both the ppERK/ERK ratio and the levels of phosphorylated FGFR1 (pFGFR1) were significantly reduced in *Cdh2* null iPSM tissues compared with the wild-type controls (Fig. 23B-D). These results confirmed our hypothesis that the Fgf signaling instead of Notch activity was disrupted by deleting *Cdh2*, which subsequently affected the *Hes7* expression.

Since there are both a posterior-anterior descending gradient and dynamic downstream activities present in the PSM, next I examined the spatial pattern of ppERK along the axis at 108 hours post-induction using immunostaining (Fig. 23E-F). Mostly recapitulating the distribution pattern in the mouse embryo (Niwa et al., 2011), in wild-type iPSM tissues, ppERK was enriched in the posterior domain, gradually attenuated along the axis reaching the trough in the anterior end where cells commit to somite fate, and increased again in the newly formed somite-like cells (Fig. 23E). By contrast, *Cdh2* KO tissues exhibited lower ppERK levels along the axis overall close to the trough intensity at anterior end, suggesting attenuated FGF signaling strength. Conversely, *Cdh2* overexpression elevated ppERK level across the tissues especially near the anterior differentiation front, flattening the normal posterior-anterior gradient (Fig. 23F). These observations indicate that *Cdh2* positively regulates the activity and distribution of the FGF/ppERK signaling pathway in PSM cells.

To specifically determine whether this observation of disrupted FGF activities is caused by impaired interaction between *Cdh2* protein and FGFR1, two complementary approaches were designed to interfere their interaction without completely removing the protein and losing homophilic adhesion (Fig. 24A, 25A). It was previously

suggested that a sequence on the extracellular domain 4 of Cdh2 protein is responsible for physical interaction with the HAV domain of FGFR1 protein (Kon et al., 2019; Williams et al., 2001, 1994) to sustain the FGF downstream activation. In order to modulate this interaction, we synthesized a blocking peptide of a sequence referring to the previous studies (Fig. 24A) and, in another case, generated a mutant cell line with the extracellular domain 4 (ECD4) of Cdh2 removed in-frame to keep other functions of Cdh2 protein (Fig. 25A). By using these 2 models, we intended to test whether the FGF signaling can be also affected. With the blocking peptide added to the culture of iPSM tissues induced from wild-type mESC cells, the overall tissue morphology was indistinguishable from the tissues in the control group with only DMSO added (Fig. 24B-D), indicating that the homophilic adhesion is not disrupted. By contrast, the ppERK levels assessed by immunostaining showed significant reduction coupled with decreased proportion of Tbx6-positive PSM cells (Fig. 24B, E-F), suggesting an antagonistic effect on FGF signaling activity of this peptide which also promoted the differentiation of iPSM cells. In addition, the evaluation of Cdh2 ECD4-deleted mutant iPSM tissues demonstrated similarly reduced ppERK levels and Tbx6-positive cell proportion without disturbing tissue architecture (Fig. 25B-E). Taken together, these results support a model in which Cdh2 likely interacted physically with FGFR1 at the cell membrane, thereby enhancing FGFR1 phosphorylation and sustaining ERK activation in PSM cells. Active form of ERK, in turn, is required to maintain Hes7 expression and oscillations and to delay the transition of PSM cells into a somite fate. When Cdh2 levels are reduced or its interaction domain is blocked, FGFR1 activation falls, whereas elevated Cdh2 stabilizes FGFR1 signaling, resulting in high ppERK levels and prolonged Hes7 oscillation.

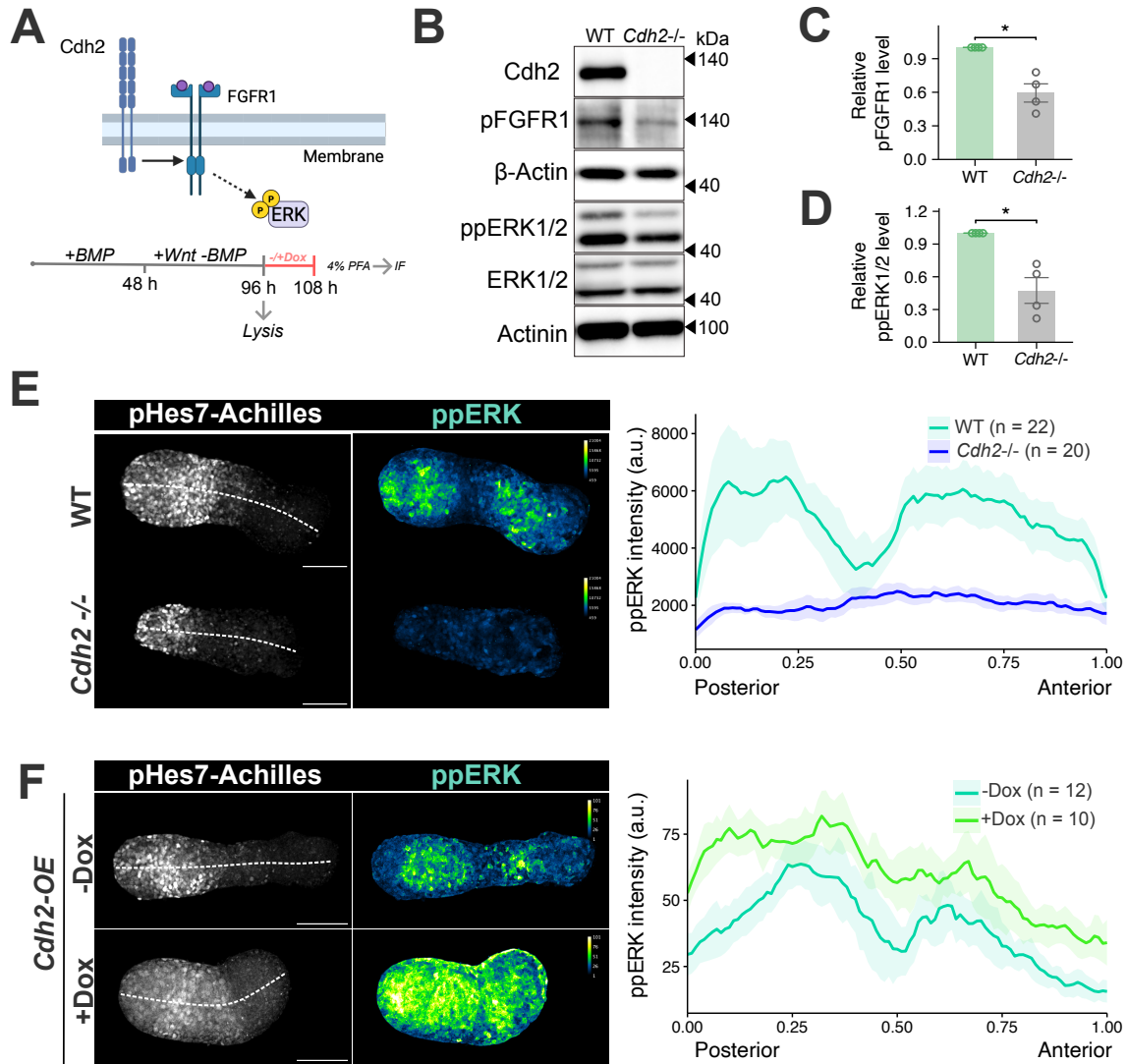


Figure 23. FGF-ppERK activity is related to Cdh2 expression levels. (A) Illustration of the relationship between Cdh2 and FGF signalling (top) and protocol for measuring ppERK in iPSM tissue by western blotting and immunofluorescence staining. (B–D) pFGFR1 and ppERK levels were quantified by western blotting. WT and *Cdh2*^{-/-} iPSM samples were collected at 96 h. Data are shown as the mean ± SEM, **p* ≤ 0.05. (E) Representative immunofluorescence images (left) and plots (right) comparing ppERK spatial profiles between WT control and *Cdh2*^{-/-} iPSM tissues, plotted on a normalized major axis where 0 is the posterior end and 1 is the anterior end. (F) Representative

immunofluorescence images (left) and plots (right) comparing ppERK spatial profiles between control (-doxycycline) and *Cdh2*-overexpressing (+doxycycline) iPSM tissues. Scale bars: 100 μm . a.u., arbitrary units.

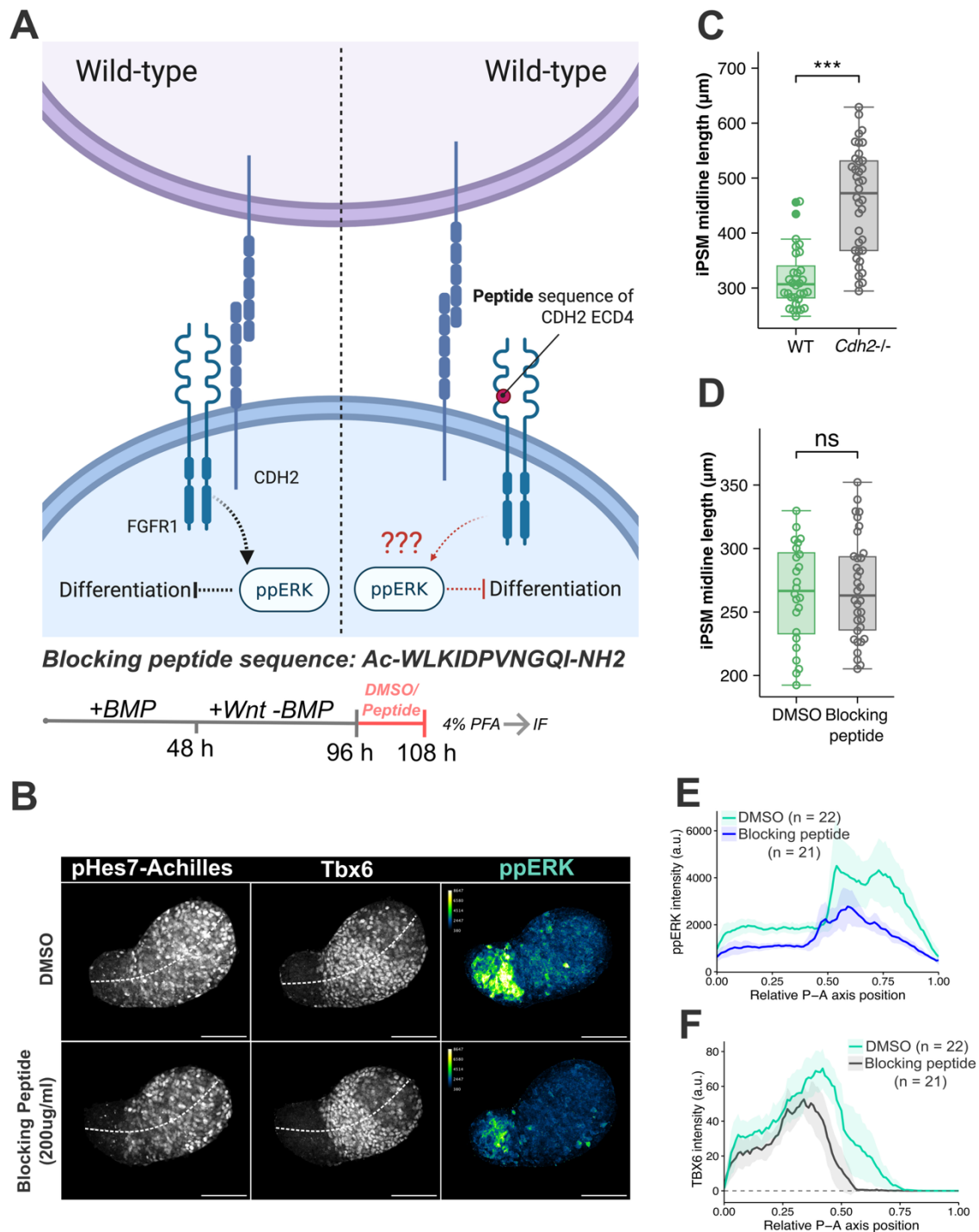


Figure 24. Effects of interference of Cdh2-FGFR1 interaction on FGF-ppERK activity and PSM cell differentiation. (A) Illustration of the experimental design for blocking peptide assay. (B,E,F) Representative immunofluorescence images (B) and plots comparing ppERK (E) and Tbx6 (F) spatial profiles between applying DMSO and

200 $\mu\text{g/ml}$ blocking peptide, plotted on a normalized major axis (dotted lines in B) where 0 is the posterior end and 1 is the anterior end. Scale bars, 100 μm . a.u., arbitrary units. (C,D) Quantification of iPSM sizes by measuring the midline length along the posterior-anterior axis, mean $\pm$ SEM. T-test: *** $p \leq 0.001$, ns: not significant.

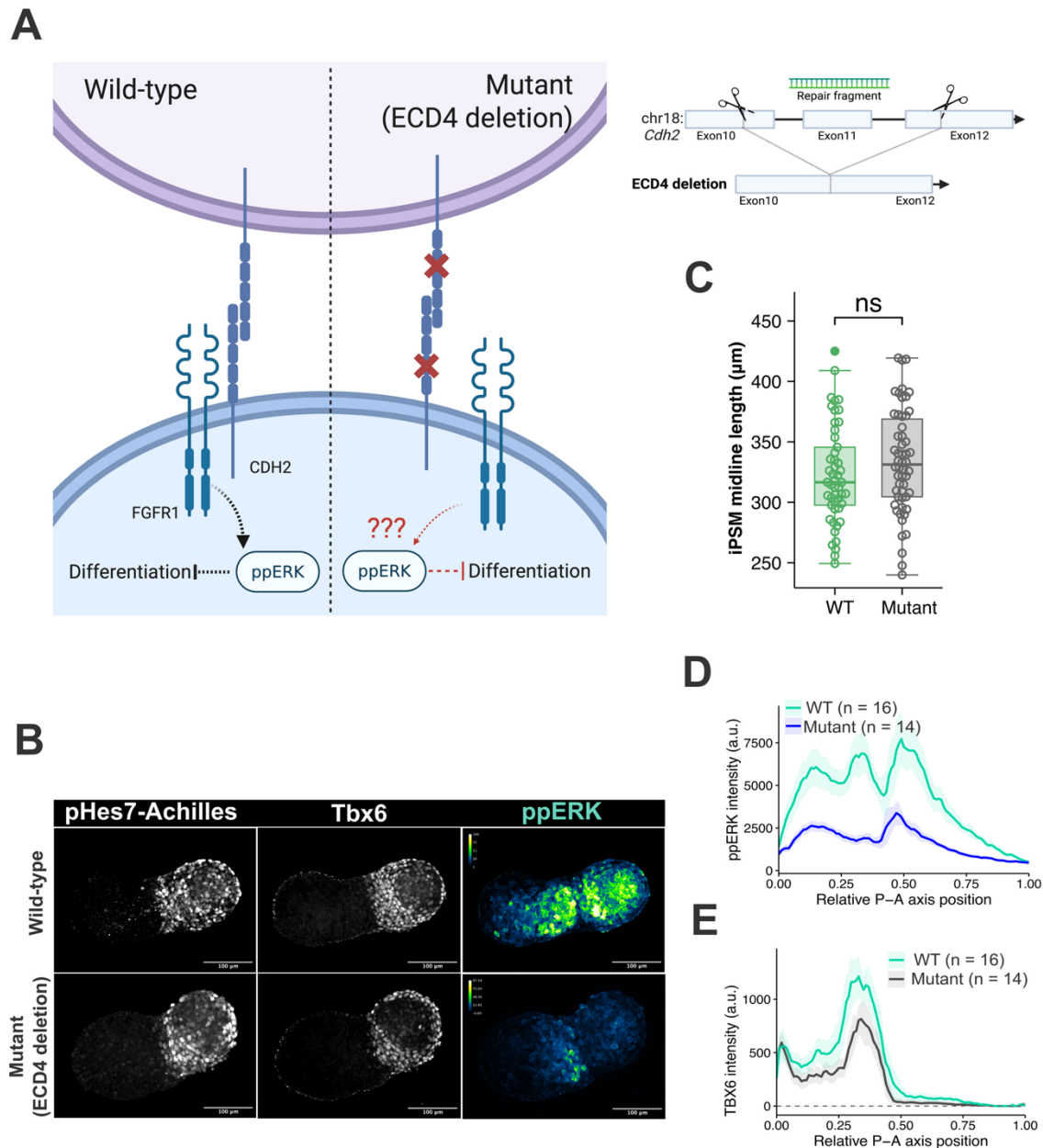


Figure 25. Effects of interference of *Cdh2*-FGFR1 interaction on FGF-ppERK activity and PSM cell differentiation. (A) Illustration of the experimental design of generating a *Cdh2* mutant with only ECD4 removed. To remove the genomic sequence corresponding to amino acid 515-604 in *Cdh2* protein, 2 sgRNA (Table 2) for targeting the 5' and 3' regions of deletion were transfected to the *pHes7*-Achilles mESCs together with a repair fragment designed equivalent to the sequence after removing the targeted region. (B,D,E) Representative immunofluorescence images of ppERK

and TBX6 (B) and plots comparing ppERK (D) and TBX6 (E) spatial profiles between wild-type and ECD4 deletion mutant, plotted on a normalized major axis where 0 is the posterior end and 1 is the anterior end. (C) Quantification of iPSM sizes by measuring the midline length along the posterior-anterior axis, mean $\pm$ SEM. T-test: ns: not significant.

3-5 Cdh2 expression influences Mesp2 patterning and presomitic mesoderm cell maturation

Cells in the presomitic mesoderm (PSM) undergo a gradual transition from an immature, oscillatory state in the posterior to a differentiated state in the anterior. This maturation process is tightly regulated by the posterior-to-anterior gradient of FGF signaling, which maintains immaturity in the posterior and permits differentiation at the anterior (Anderson et al., 2020; Dubrulle et al., 2001; Sawada et al., 2001; Wahl et al., 2007). The frequency of the clock is also coupled with this spatial gradient, where the oscillatory expression wave slows as it reaches the anterior side (Niwa et al., 2011; Shih et al., 2015; Tsiairis and Aulehla, 2016; Yaman and Ramanathan, 2023). A key marker of this transition is the activation of *Mesp2*, a transcription factor expressed in a stripe-like on-off manner within the anterior PSM that specifies the future somite boundaries (Morimoto et al., 2005; Niwa et al., 2011; Oginuma et al., 2008). Since FGF signaling represses *Mesp2* expression, alterations of FGF/ppERK activity observed previously in *Cdh2* mutants are expected to influence the PSM maturation and *Mesp2* expression dynamics.

To examine how *Cdh2* levels affect the states of PSM cell maturation, I compared the periods of *Hes7* oscillations between wild-type (WT) and *Cdh2* KO iPSM tissues (Fig. 26A), which can indicate the maturity states of the cells. The periods extracted from oscillations were measured from regions of interest (ROIs) by calculating the phase-to-phase intervals. In the WT tissues, the average of oscillation periods extracted from the posterior iPSM side was shorter compared to more anterior regions (Fig. 26B), reflecting more maturation in the anterior cells. In *Cdh2* KO tissues, however, the periods from posterior regions were close to the length normally seen in

the anterior of WT tissues. Generally, there is more maturity at the tissue level when *Cdh2* is absent, while it is unclear if any tissue integrity and collective migration alterations contribute to these long periods.

To further test whether this slowing was not only a tissue-level effect but also an intrinsic cellular property, I also measured and compared periods at single-cell level by dissociating iPSM into single-cell cultures and tracking the *pHes7*-Achilles reporter oscillations in each single cell (Fig. 26C). By pooling the periods extracted from tracks together, it confirmed that *Cdh2* KO cells had slower oscillations compared with WT cells (Fig. 26D). These findings together suggest that loss of *Cdh2* intrinsically promotes anterior-like properties on PSM cells.

To investigate whether the *Mesp2* expression patterns were altered by the pre-maturation in PSM cells, I compared *Mesp2* mRNA distribution in *Cdh2* KO and *Cdh2* overexpression iPSM tissues with wild-type. In WT iPSMs, *Mesp2* was expressed as a sharp strip at anterior, corresponding to the region where *Hes7* oscillation stops (Fig. 27A-B, WT). There are fluctuations of intensity among the samples, indicating the *Mesp2* expression is dynamic in the differentiation front. However, in *Cdh2* KO samples, the *Mesp2* expressing strips were shifted posteriorly with higher levels, which is consistent with the observations of accelerated differentiation (Fig. 27A-B, *Cdh2*^{-/-}). By contrast, in *Cdh2* overexpressing tissues, the strips were shifted more anterior and expressed at a lower intensity, indicating delayed differentiation (Fig. 27A-B, *Cdh2*-OE). The quantification confirmed these positional changes, supporting the conclusion that *Cdh2* levels regulate the timing and level of *Mesp2* activation and PSM cell maturation.

To analyze the dynamics of *Mesp2* expression with more details, I measured peak intensity (signal amplitude) and stripe width (spatial range). In *Cdh2* KO iPSM tissues, both amplitude and range were increased relative to WT (Fig. 27C-D), suggesting an extended anterior-like domain in the posterior part. Notably, the fluctuations of *Mesp2* intensity were also visible in KO conditions (Fig. 27C-D). These findings indicate that in the absence of *Cdh2*, *Mesp2* expression not only appears earlier but also spreads over a broader region, potentially due to reduced Fgf signaling activity and permitted pre-maturation. In contrast, in *Cdh2* overexpressing tissues, *Mesp2* expression was much weaker with overall lower peak intensities, shortened spatial range and the differences among samples were less visible (Fig. 27C-D), suggesting that elevated *Cdh2* suppressed *Mesp2* expression dynamics.

Together, these data show that *Cdh2* levels regulate PSM cell maturation by influencing both the timing and the dynamics of *Mesp2* patterning (Fig. 27E). Loss of *Cdh2* leads to early differentiation and a posterior expansion of somite marker expression which is revealed by *Uncx4.1* (Fig. 28A,B) and *Meox1* (Fig. 28C,D) expression, while high level of *Cdh2* shifts *Mesp2* anteriorly and delays differentiation. Thus, proper reduction of *Cdh2* levels and subsequent repressed FGF activity at the anterior PSM is essential for timing and positioned *Mesp2* activation and boundary formation (Fig. 27F). It also revealed how cell adhesion molecules integrate with signaling gradients to fine-tune the differentiation thresholds. The ability of *Cdh2* to shift the balance between immaturity and commitment suggests that adhesion molecules also participate directly in setting the position and timing of segmentation.

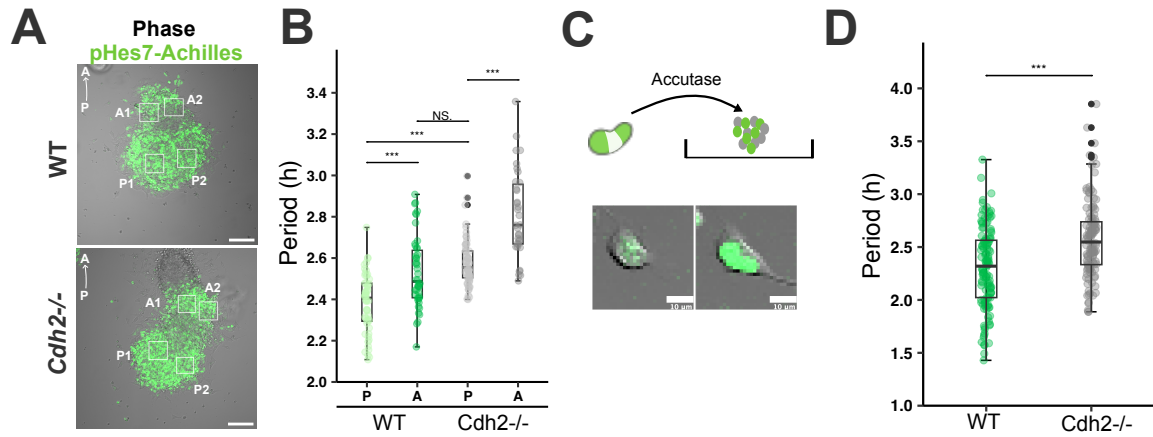


Figure 26. The *Hes7* oscillations slowdown in *Cdh2* KO iPSM tissue. (A-B) Oscillation periods of fluorescence of the p*Hes7*-Achilles reporter in iPSM tissue. The periods were exacted from the ROI (A) plot by calculating the peak-to-peak intervals. A: ROI at the anterior iPSM; P: ROI at the posterior iPSM. ANOVA: **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, NS: not significant.(C) iPSM tissues were cultured following the iPSM protocol. At 96 h, iPSM tissue was dissociated into single cells using Accutase and plated on fibronectin-coated plates. Scale bars, 10 μm. (D) Oscillation periods of fluorescence of the p*Hes7*-Achilles reporter in dissociated iPSM cells. Error bars indicate SEM. t-test: ****p* ≤ 0.001.

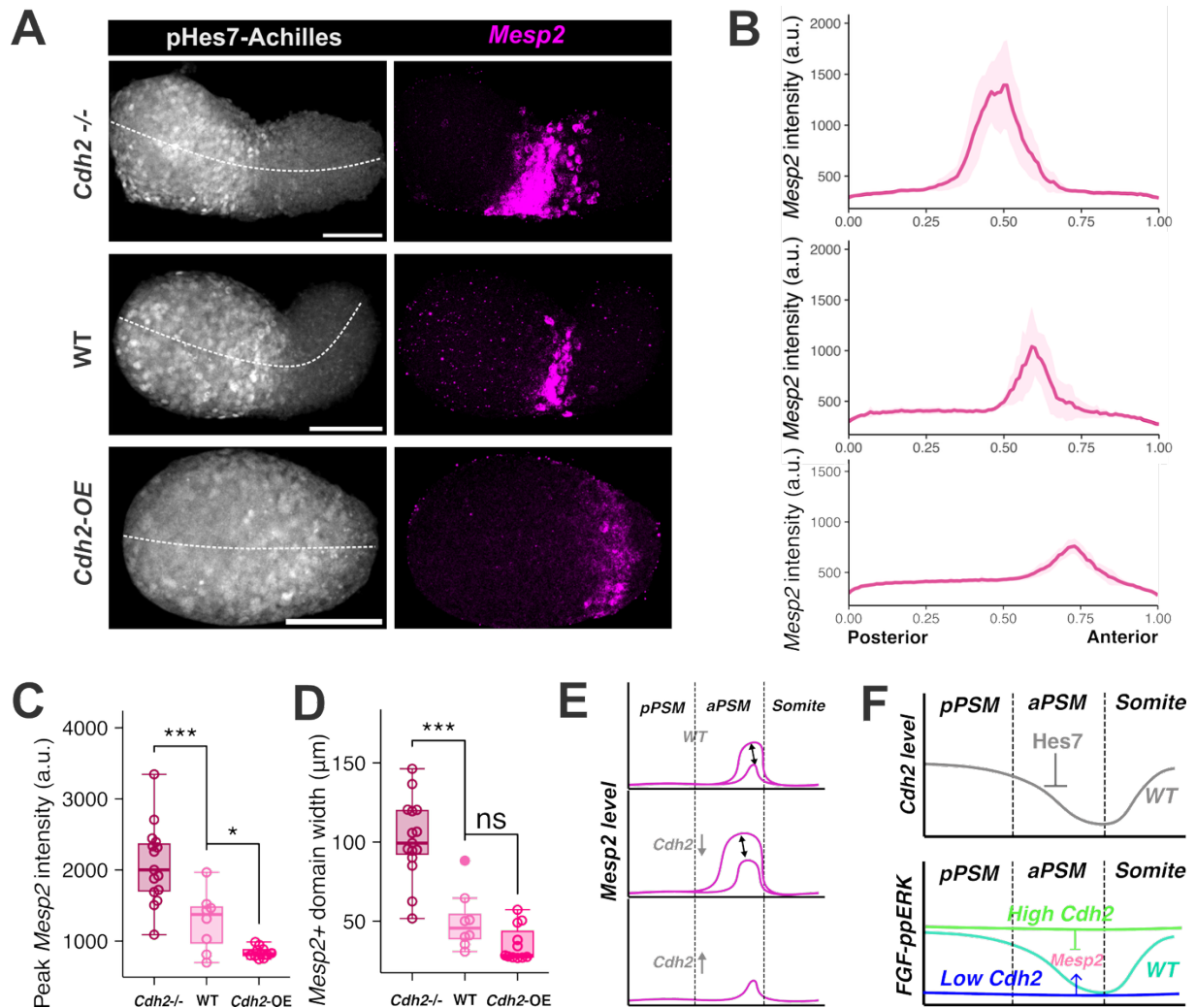


Figure 27. *Cdh2* KO iPSM tissues gained more anterior like properties. (A) Representative hybridization chain reaction staining images of *Mesp2* mRNA expression (magenta) and pHes7-Achilles activity (grey). Scale bars, 100 μm. (B) Spatial plot of *Mesp2* mRNA expression along the posterior-anterior axis. Data are the mean ± 95% confidence interval, with the 2.5th and 97.5th percentiles shown for each data point. (C) Maximal signal intensity of *Mesp2* mRNA expression extracted from the plots (B). (D) The spatial length of *Mesp2* mRNA expression in iPSM tissue calculated from the plots (B). Mean ± SEM. t-test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS: not significant. (E) Scheme summarizing the phenotypes of *Mesp2* expression. The magenta lines represent the expression intensity along the axis, and the black arrow

headed lines between the magenta lines were used to indicate the fluctuations between samples. (F) Summary of perturbations of *Cdh2* expression in iPSM tissue and the resultant phenotypes.

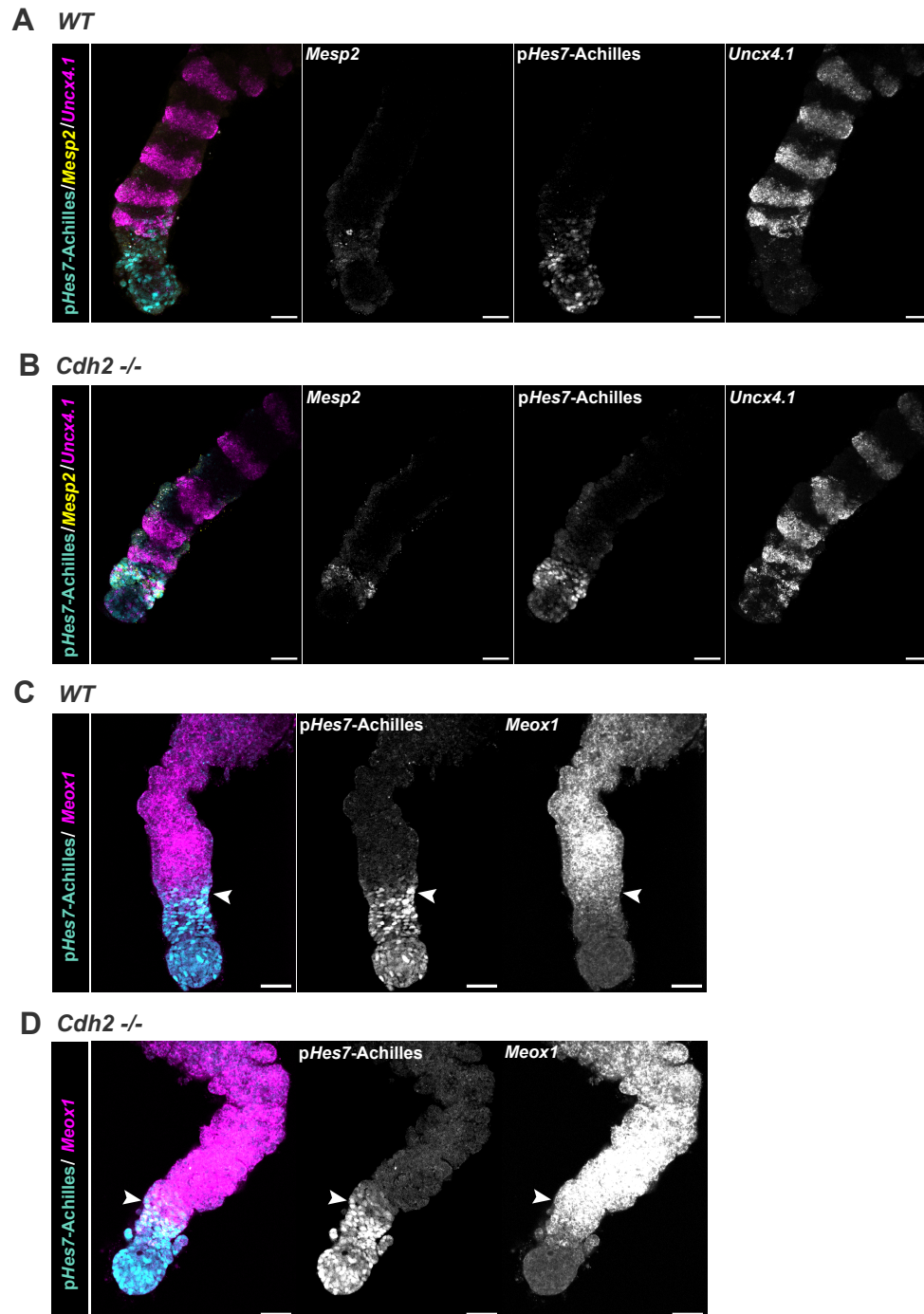


Figure 28. Hybridization chain reaction staining to examine somite formation.

(A,B) iPSM tissue was embedded in 10% Matrigel/ Ndiff227 at 96 h and grown for an additional 20 h before fixation. iPSM tissue was then removed from Matrigel and

stained for *Uncx4.1* (magenta) and *Mesp2* (yellow). (C,D) iPSM tissues in the same condition as above are stained for *Meox1* (magenta). Scale bars: 50 μm .

Chapter 4

Discussion

4-1 Aim and Summary of this thesis

The oscillatory expression of Hes7 serves as a fundamental regulatory mechanism in vertebrate somitogenesis, ensuring the precise coordination of temporal and spatial cues during segment formation. Over recent decades, accumulating studies have revealed intricate interactions between Hes7 and several key signaling pathways, including Notch, FGF, and Wnt, suggesting that Hes7 acts as a central integrator linking the segmentation clock to gradient-based positional information. However, despite these advances, the molecular mechanisms through which Hes7 controls downstream targets and influences signaling feedback remain incompletely understood. Therefore, this thesis aims to elucidate the downstream regulatory network of Hes7 in the mouse presomitic mesoderm (PSM), to identify direct Hes7 target genes and examine their roles in oscillatory regulation, tissue organization, and signaling integration within the segmentation clock framework.

In summary, this study expanded the regulatory network of the oscillatory transcriptional factor Hes7 and identified a regulatory connection between Hes7 and the adhesion molecule *Cdh2*. By periodically repressing *Cdh2* transcription, Hes7 generated shallow waves of *Cdh2* mRNA expression in the PSM. Because Cdh2 protein is relatively stable compared with Hes7, these fluctuations are translated into a graded posterior-anterior protein distribution, rather than oscillations. High Cdh2 expression at the posterior PSM maintains high Fgf/ERK activity therefore immature PSM identity and the drop of its levels at anterior regions coupling the reduced FGF activity permits *Mesp2* expression and PSM cell to differentiate and commit into somite fate. Following somite formation, *Cdh2* expression is restored ensuring somite structure integrity through adhesion. Thus, Hes7 regulates somitogenesis over a broad

range of downstream genes by integrating oscillatory transcriptional programs with diverse cellular components and signaling environments, linking temporal control to cell differentiation processes and morphogenetic outcomes.

4-2 Expansion of Hes7 regulatory landscape and bridging the clock and the signaling gradients

The genome-wide ChIP-seq revealed that Hes7 regulates a much broader set of targets than previously recognized. In addition to classical clock-related genes such as *Lfng* (Bessho et al., 2003) and *Dusp4* (Niwa et al., 2011), many novel candidates were identified involved in biological processes such as cell proliferation, cell death, cellular component organization, cell migration and others (Fig. 8E), implicating Hes7 in wider regulatory networks in the PSM. Interestingly, Hes7 did not bind to the *Dll1* locus, in contrast to zebrafish *her1/her7* proteins that directly regulate *deltaC* (Oates et al., 2012), which explains that this association in mice has not been reported so far and suggests that mammalian Hes7 regulates Notch signaling mainly via *Lfng*. Such differences highlight the evolutionary diversity of segmentation clock networks, even when their overall logic remains conserved. Among the Hes7 targets, though only *Cdh2* emerged controlling oscillation, some other targets also showed importance for PSM viability and identity which requires more investigation.

Previous research has demonstrated that FGF signaling exhibits two distinct dynamic modes within the PSM: a spatial gradient of ligand distribution extending from the posterior to anterior regions (Dubrulle et al., 2001; Dubrulle and Pourquié, 2004), and temporal oscillations of activation, reflected by periodical changes in dual phosphorylated ERK (ppERK) levels (Delfini et al., 2005; Niwa et al., 2011, 2007;

Simsek et al., 2023). The loss of these oscillations in *Hes7*-null embryos disrupts the regular segmentation, resulting in abnormal somite patterning in mice. Comparable phenotypes have been reported in *her1/her7*-deficient zebrafish embryos, where restoring periodical FGF/ppERK inhibition reestablishes proper boundary formation (Simsek et al., 2023). Among the known regulators, *Dusp4*, a phosphatase that antagonizes ppERK activity, has been identified as a direct transcriptional target of *Hes7* (Niwa et al., 2011, 2007), which is also confirmed by the ChIP-seq cohort in this study. Nevertheless, due to functional redundancy and compensatory feedback within the FGF signaling network, *Dusp4*-deficient mice have not conclusively revealed how *Hes7* modulates this pathway (Auger-Messier et al., 2013).

In this study, ChIP-seq analysis identified *Cdh2* as another *Hes7*-related gene, providing new insight into how *Hes7* might influence ppERK dynamics indirectly. Previous work in other cellular systems demonstrated that *Cdh2* interacts with the extracellular domain HAV of FGFR1, thereby modulating FGF signaling and downstream ERK phosphorylation (Kon et al., 2019; Suyama et al., 2002; Takehara et al., 2015; Williams et al., 2001, 1994). These findings, together with our observations, suggest that *Hes7*-mediated repression of *Cdh2* expression could act as a mechanism to fine-tune FGF signaling activity in PSM. This places *Cdh2* as another bridge between *Hes7* oscillations and FGF signaling, explaining how regulation of an adhesion molecule can indirectly fine-tune the gradient and supporting ppERK oscillations.

4-3 Adhesion balance, synchronization and tissue organization

Another key observation was the discrepancy between dynamic *Cdh2* mRNA expression and the stable Cdh2 protein gradient. While *Cdh2* transcripts exhibited shallow wave-like oscillations coupled to Hes7, the protein formed a posterior-high to anterior-low gradient. This is later explained by the difference in the turnover rates where Hes7 protein is short-lived (~20 min), whereas Cdh2 persists for hours. Such buffering of oscillatory inputs into stable outputs gives another example of clock gene regulatory networks, instead of generating an oscillatory expression pattern, the clockwise information is translated into spatial signals to connect the clock and the signaling gradients.

Although Cdh2 did not appear essential for Notch activity with NICD levels unchanged in KO tissues, subtle effects were detected upon overexpression with reduced NICD and Lfng levels. Since Lfng is indispensable for synchronization of Hes7 oscillations (Okubo et al., 2012; Yoshioka-Kobayashi et al., 2020), the shifts in its expression may explain the weakened synchrony of Hes7 oscillation in the Cdh2 overexpressing tissues. Moreover, Cdh2 can recruit presenilin1 (Marambaud et al., 2003), linking it to the potential to influence Notch receptor cleavage. These observations suggest that while Cdh2 may mainly play a role in Fgf signaling regulation, it may also modulate Notch signaling and oscillatory synchrony through other mechanisms. In addition, the excess Cdh2 disrupted axis direction inside the iPSM tissues, indicating that beyond signaling, changes in Cdh2 levels on cell membranes also affect collective migration and cell sorting during tissue morphogenesis. In our data where Hes7 was overexpressed, imbalanced Cdh2 triggered sorting between cells with high Cdh2 levels and low levels (Fig. 13E). These findings imply that a homeostatic balance of adhesion strength is maintained in the PSM, allowing cells to

move, oscillate, and differentiate in a coordinated manner. Hes7 repression of Cdh2 may therefore serve dual purposes to tune signaling pathways and orchestrate the cell behaviors in segmentation clock.

4-4 Methodological advances

To deepen the mechanistic understanding of clock gene function, this study sought to optimize in vitro approaches that allow direct and systematic manipulation of segmentation clock components. Although the previously established iPSM culture system carrying a *pHes7* reporter has been successfully employed for chemical screening and temporal analysis of oscillatory dynamics (Matsumiya et al., 2018; Yoshioka-Kobayashi et al., 2020), its application in dissecting specific molecular interactions has remained limited due to the complexity of signaling pathways within the system. Here, we integrated the iPSM platform with CRISPR/Cas9-mediated gene knockout and inducible overexpression techniques, establishing a more flexible and precise framework for testing candidate regulators of the segmentation clock. The system further enables real-time live imaging of reporter activity at both tissue and single-cell resolution, providing simultaneous access to temporal oscillatory patterns and spatial organization. Collectively, this methodological advancement offers a powerful and versatile platform for functionally decoding the genetic and signaling networks that sustain rhythmic gene expression and somite patterning, thereby accelerating the identification of new regulatory components in vertebrate somitogenesis.

4-5 Limitations and future perspectives

While this study establishes a connection between Hes7, Cdh2 and FGF/ERK signaling, several limitations remain. We revealed that Hes7 binds to the *Cdh2* locus through ChIP-seq and represses its transcription to adjust its protein levels (Fig. 13D-E), the necessity of this binding site has not been directly tested. Targeted mutagenesis of the binding motif would clarify this. In addition, although our data supports Cdh2-FGFR1 interaction, its structure basis in PSM cells is unknown and co-immunoprecipitation and structure assays will be required to provide more information. It is also intriguing to understand the detailed mechanistic mechanism of how Cdh2-FGFR1 interaction facilitates the stable FGFR1 phosphorylation to prevent its internalization from cell membrane. Moreover, altered adhesion may affect cell mechanics, which have been implicated in segmentation clock function (Hubaud et al., 2017; Sung et al., 2024 in press). Mechanical measurements should be integrated into future analyses. Future studies combining genetic, biochemical and biophysical approaches will be required to resolve these open questions.

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本学位論文は以下の学術論文の内容に基づいて書かれたものである。

Xueqi Jia, Akihiro Isomura, and Ryoichiro Kageyama

**Cdh2, a downstream target of Hes7, regulates somitogenesis by supporting
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