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The Essential Roles of the Chromatin Factor Gon4l in Heart Development

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The Essential Roles of the Chromatin Factor Gon4l in

Heart Development

by

Terin Elise Budine

A dissertation presented to
The Graduate School
of Washington University
in partial fulfillment of the
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List of Abbreviations

μ	microgram
μL	microliter
μm	micrometer
μM	micromolar
ALPM	anterior lateral plate mesoderm
amhc	*atrial myosin heavy chain*
AV	atrioventricular
BMP	bone morphogenetic proteins
BSA	bovine serum albumin
CHDs	congenital heart defects
CPCs	cardiac progenitor cells
ccnd	*cyclin d*
ccne	*cyclin e*
cmcl2	*cardiac myosin light chain 2*
Co-IP	co-immunoprecipitation
DamID	DNA adenine methyltransferase identification
DAPI	2-(4-Amidinophenyl)-1H-indole-6-corboxamidine
dpf	days post fertilization
DMSO	dimethyl sulfoxide
DV	dorsal-ventral

EdU	5-ethynyl-2'-deoxyuridine
ESCs	embryonic stem cells
hESCs	human embryonic stem cells
FGF	fibroblast growth factors
G1	growth 1
G2	growth 2
GFP	green fluorescent protein
gsc	*goosecoid*
GO	gene ontology
Gon4l	Gonad-4-like
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
hpf	hours post fertilization
hsp	*heat shock promoter*
kny	*knypek*
LPM	lateral plate mesoderm
LSK	Lilianna Solnica-Krezel
MCM	minichromosome maintenance complex
MO	morpholino
myl7	*myosin light chain 7*
myh6	*myosin heavy chain 6*
myh7	*myosin heavy chain 7*

MZ	maternal-zygotic
ng	nanogram
PBS	phosphate buffered saline
PBT	phosphate buffered saline with tween
PCP	planar cell polarity
PCR	polymerase chain reaction
PFA	paraformaldehyde
qRT-PCR	quantitative real-time PCR
RA	retinoic acid
s	somite
SANT	Swi3, Ada2, Cor, and TFIIIB
S-phase	synthesis phase
Tg	transgene
tp53	*tumor protein 53*
TGFβ	transforming growth factor beta
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling
udu	*ugly duckling*
vmhc	*ventricular myosin heavy chain*
WISH	whole-mount *in situ* hybridization
WT	wild type
Yy1	*Ying-yang 1*

YY1AP   *Ying-yang 1 Associated Protein*

Z   zygotic

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Terin Elise Budine

Washington University

December 2018

Dedicated to my parents and Ryan

Except Ryan told me to dedicate it to the cats

So, dedicated to my parents, Starr, and Theo

For their unconditional love and support

ABSTRACT OF THE DISSERTATION

The essential roles of the chromatin factor Gon4l in

heart development

by

Terin Elise Budine

Doctor of Philosophy in Biology in Biomedical Sciences

Developmental, Regenerative, and Stem Cell Biology

Washington University in St. Louis, 2018

Professor Lilianna Solnica-Krezel, Advisor

Professor Kristen Kroll, Chair

Heart development and the genetic pathways underlying it are highly conserved among
vertebrates. During heart development, an embryo must induce mesoderm formation, pattern the
mesoderm, specify cardiomyocytes, increase the population of cardiomyocytes through
proliferation, and pattern the cardiac chambers. It is becoming increasingly clear that chromatin
modifications help mediate the complex processes of heart development by providing
spatiotemporal regulation of gene expression. My thesis work focuses on characterizing
functions of the chromatin factor Gonad-4-like (Gon4l), encoded by the gene *ugly duckling*
(*udu*), in zebrafish heart development. Previous works established a requirement for Gon4l in the
formation of many mesoderm derivatives including somites and blood. My studies define novel
roles for maternal and zygotic Gon4l function in zebrafish heart development. Embryos lacking
both maternal and zygotic *udu* (MZ*udu*) expression have perturbations in the formation of all

three germ layers, but this thesis focused on the disruptions in mesoderm development. MZ*udu*$^{-/-}$ embryos have abnormal mesoderm patterning that results in defects in the development of mesoderm derived tissues and organs. MZ*udu* mutant embryos present an almost complete loss of anterior lateral plate mesoderm formation, and subsequently heart development. RNA-sequencing performed on MZ*udu*$^{-/-}$ embryos uncovered broad misregulation of genes including morphogens and transcription factors. Cell autonomy transplants indicated that Gon4l has both cell-autonomous and non-cell-autonomous functions in heart development, which suggests that disruption of multiple signaling pathways contributes to the defects in heart development. In addition, this work establishes separate zygotic functions for Gon4l in cardiomyocyte proliferation and maintenance of ventricular identity. Notably, these deficiencies in heart development are not due to increased expression of *tp53*, unlike the hematopoiesis defects in zygotic *udu* (Z*udu*) mutant embryos. Cardiomyocyte proliferation is reduced in Z*udu*$^{-/-}$ embryos. Differential expression of *ccnd3* and *ccne2* and evidence that Gon4l associates with regulatory regions of these *cyclins* posits that Gon4l may be required for progression through the G1/S cell cycle checkpoint. Analysis of *nkx2.5;udu* compound mutant phenotypes revealed that *nkx2.5* and *udu* genetically interact in the maintenance of ventricular identity. However, ectopic expression of *nkx2.5* failed to restore ventricular patterning in *udu*$^{-/-}$ embryos, which indicates that Gon4l could regulate multiple pathways to maintain ventricular identity. Altogether my work establishes novel roles for Gon4l in regulating mesoderm patterning, cardiomyocyte proliferation, and maintenance of ventricular identity during vertebrate heart development.

Chapter 1: Introduction

1.1 Congenital Heart Defects

The heart is the first organ to form and function in the developing vertebrate embryo. Human embryos have a beating heart tube by day 21 of development. Unfortunately, cardiac development is prone to errors. Many of these errors are incompatible with survival of the fetus or lead to congenital heart defects (CHDs), which are the most common human birth defect affecting approximately one percent of newborns (Pierpont et al., 2007). The cost of CHDs in monetary terms and in human suffering is enormous.

The majority (72%) of CHDs have no known genetic cause (Russell et al., 2018). Defects during any stage of heart development from mesoderm specification and patterning to the later maturation of the heart can result in CHDs. Treatment has traditionally been surgery or transplantation of a donor heart (Agrawal et al., 2017), and these interventions carry risk to the patient as well as long-term follow-up care (Seghaye, 2017). New methods, which take advantage of improved cell differentiation methods, offer the potential of tissue-engineering defect valves or using cardiomyocytes derived from stem cells to regenerate parts of the heart (Seghaye, 2017). To better diagnose and treat CHDs and to improve methods to generate cardiomyocytes in culture, it is essential to identify the genes that regulate cardiac development. The developmental networks underlying heart development are complex and require myriad proteins including secreted molecules, cell-surface molecules, and transcription factors. Advances in understanding heart development point to epigenetic regulation of the genes being

involved in these critical process of cardiogenesis. Therefore, developing new methods for treating and diagnosing CHDs requires understanding the role chromatin factors in heart development.

Zebrafish are an excellent model with which to study CHDs. Heart development is rapid: by 24 hours post fertilization (hpf), the embryos have a beating heart tube (Bakkers, 2011). The small size of the embryo allows for passive diffusion of oxygen and survival up to 5 days post fertilization (dpf) without a functioning cardiovascular system. This physiology makes it possible to study even the most severe cardiac defects. Despite the morphological differences - the zebrafish heart containing only two chambers compared to the four chambers of the mammalian heart - the genetic and morphogenesis programs that specify and shape the heart are highly conserved across vertebrates (Figure 1.1). The amenability of the zebrafish to genetic manipulation and forward genetic screens make it an ideal system to identify and investigate novel regulators of heart development.

This dissertation focuses on the role of the chromatin factor Gon4l in heart development. Our studies indicate novel roles for Gon4l at many stages of heart development from the earliest patterning of the mesoderm through cardiomyocyte proliferation and patterning of the heart chambers. Results suggest that Gon4l is essential for zebrafish heart development and it helps mediate many developmental processes and signaling networks.
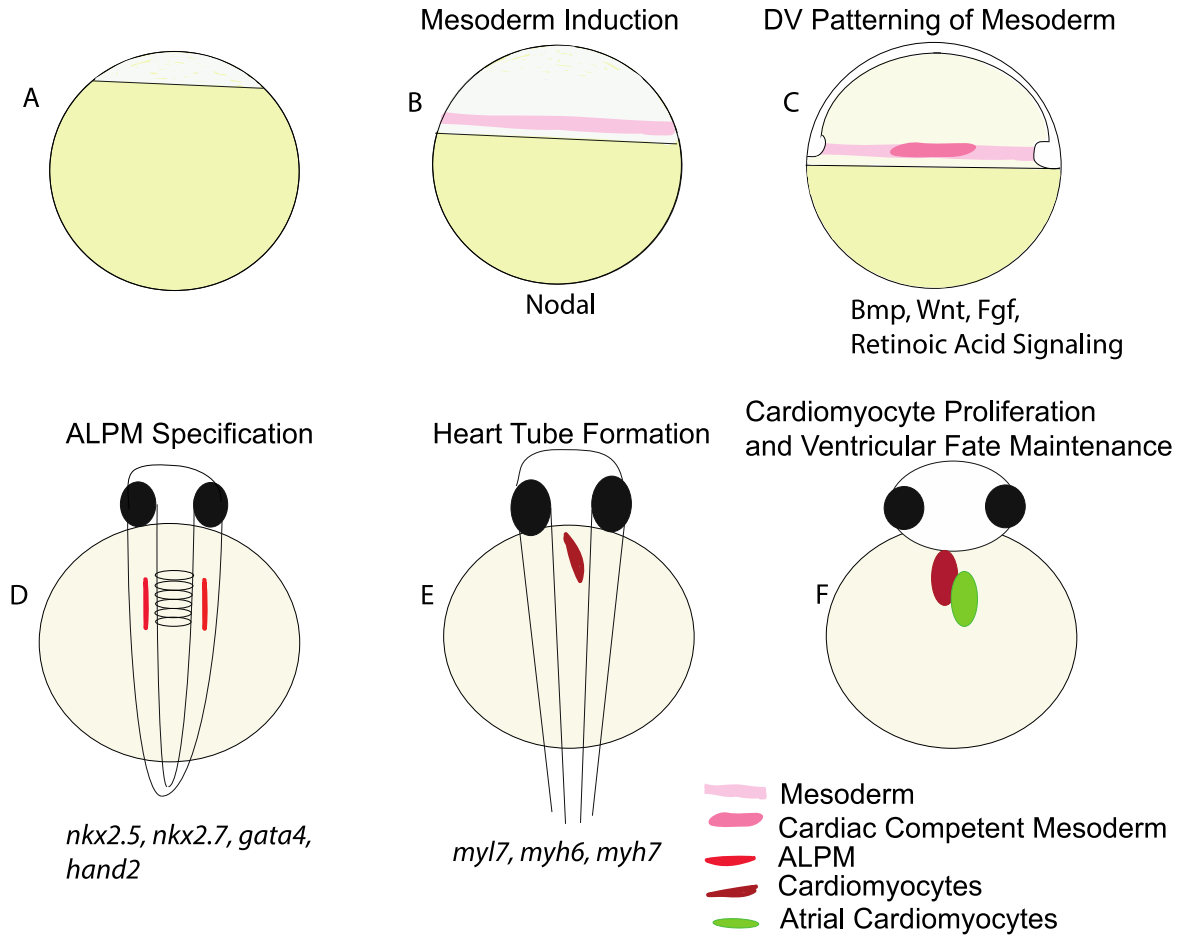
Figure 1.1 Heart development in zebrafish

A. One-cell stage zebrafish embryo

B. Mesoderm induction in zebrafish embryo at sphere stage

C. Dorsal-ventral patterning of the mesoderm at shield stage

D. Anterior lateral plate mesoderm specification at 6-somite stage

E. Heart tube formation at 24 hpf

F. Zebrafish heart at 48 hpf with cardiac chambers patterning

1.2 Mesoderm specification and patterning

One of the first tasks of an embryo during development is specifying the three germ layers, mesoderm, endoderm and ectoderm. Germ layer formation begins the process of transforming cells from their naïve totipotent state to fully specified and differentiated mature fates (Kimelman, 2006). The three germ layers establish a blueprint for the embryonic body plan and provide the material from which the organs of the body derive. Fate mapping studies have shown that at the blastula stage, preceding gastrulation, the cells of the embryo are loosely organized into the nascent germ layers, but that the cells are not yet fully specified (Kimmel, 1988). At this stage of development (4 hpf), the zebrafish blastula consists of a large syncytial yolk cell, topped with a mound of blastomeres, and the zygotic genome has been activated. The prospective endoderm is closest to the yolk. The mesoderm arises from the cells lying just above the endoderm (Figure 1.1 B). And ectoderm will derive from the cells furthest from the yolk. Initially, the future mesoderm and endoderm are co-mingled and are referred to as the mesendoderm (Warga and Kimmel, 1990).

Gastrulation is the process by which the specified germ layers move to achieve their three-dimensional arrangement and are shaped into the nascent body plan. At the start of gastrulation in zebrafish, the mesendoderm will ingress through the blastopore at the blastoderm/yolk margin in a synchronized manner (Solnica-Krezel and Sepich, 2012). Following ingression, the mesendoderm separates into two layers. The mesoderm will migrate as single-cells away from the blastopore via directed migration while endoderm cells migrate between the yolk surface and mesoderm through random-walk movements towards the animal pole (Pezeron et al., 2008; Solnica-Krezel and Sepich, 2012). After gastrulation, endoderm will be the deepest germ layer, ectoderm will be the most superficial, and mesoderm will be sandwiched between

the two (Williams and Solnica-Krezel, 2017). The endoderm is the origin layer of the gut and its derivatives including the liver and pancreas. Mesoderm differentiates into the muscles, skeleton, connective tissues, heart, kidney, and blood. The ectoderm will become the epidermis and neural tissues. By the end of gastrulation, the germ layers of the embryo have been committed to their fate and have migrated to establish the basic structure that will underlie the remainder of embryogenesis.

Specification and morphogenesis of the germ layers during gastrulation requires contributions from many signaling pathways, but, perhaps, the most important of these signaling pathways is the TGFβ superfamily (Kiecker et al., 2016). The TGFβ superfamily includes many sub-families including Nodals, Bone Morphogeneic Proteins (BMPs), Vg-1, and Activin (Kiecker et al., 2016). Broadly, TGFβ signaling acts through ligand-receptor complexes that upon ligand binding transduce signals through SMAD proteins that bind to other transcription factors and epigenetic modifiers that will initiate or maintain gene expression (Massague, 2012). Many members of the TGF-β family have critical functions during embryogenesis from germ layer specification and patterning to organogenesis (Dosch, 1997; Prall, 2007; Shen, 2007).

Studies in *Xenopus*, zebrafish, and mouse indicate that Nodal signaling is likely the main mesendoderm inducer (Conlon, 1994; Feldman, 1998; Kiecker et al., 2016). Ectopic Nodal expression in *Xenopus* and zebrafish is capable of inducing expression of mesodermal genes (Agius, 2000; Toyama, 1995). Perhaps the more convincing support for the role of Nodal signaling in mesoderm specification is the strong reduction in mesoderm formation that occurs when Nodal signaling is disrupted. In mouse, genetic disruption of Nodal signaling causes a failure of mesoderm specification and early embryonic death (Conlon, 1994; Shen, 2007; Zhou, 1993). In zebrafish, β-catenin localized in the nuclei of the dorsal yolk syncytial layer and

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blastomeres induce expression of the *nodal-related* genes *squint* (*ndr1*) and *cyclops* (*ndr2*) (Chen, 2000; Shen, 2007) dorsally and still unidentified mechanisms activate broader expression of these two genes around the blastoderm margin. Zebrafish embryos double mutant for these two genes exhibit a nearly complete loss of dorso-lateral mesoderm and endoderm specification. Embryos lacking Nodal ligands fail to express markers of axial, paraxial and intermediate mesoderm (Feldman, 1998). Furthermore, genetic loss of the transducers of TGFβ signaling *smad2*, *smad3*, and *smad4* leads to severe defects in mesoderm development (Dunn et al., 2004; Vincent et al., 2003). In summary, these experimental results indicate that Nodal signaling is required in vertebrate embryos for proper mesoderm specification.

In addition to Nodal signaling, other members of the TGF-β superfamily are capable of mesoderm induction. Experiments in *Xenopus* demonstrated the ability of mature Vg-1 protein to induce mesoderm in animal cap explants (Dohrmann, 1996; Kessler, 1995), and in zebrafish, maternal Vg-1 is required for endogenous Nodal signaling and mesendoderm induction (Montague and Schier, 2017). Other TGFβ family members including Bmps are weak mesoderm inducers when injected into animal cap explants (Kiecker et al., 2016; Nishimatsu, 1998). There remains debate regarding the ability of Bmps to induce mesoderm in animal caps; with some studies finding that injections of *Bmp-4* and *Bmp-2* into *Xenopus* animal cap explants can induce mesoderm formation and ventralize the embryo (Dale, 1992) while others suggest that the mesoderm inducing activities only occur when *Bmp2/7* and *Bmp4/7* heterodimers can form (Nishimatsu, 1998). Loss of Bmp signaling components in zebrafish perturbs, but does not block, mesoderm formation, suggesting that Bmp is not required for mesoderm induction (Ober and Schulte-Merker, 1999). Indeed, as will be described later extensive genetic and biochemical

studies in zebrafish implicate Bmp2/7 and Bmp 2/4 heterodimers in mesoderm patterning (Tuazon and Mullins, 2015).

Notably, loss of Nodal signaling and other members of the TGFβ superfamily does not result in a complete loss of mesoderm in mouse, frog, or fish, implying that other pathways are involved (Feldman, 1998; Kiecker et al., 2016; Zhou, 1993). Overexpression of Fibroblast Growth Factor (Fgf) signaling is competent to induce mesoderm in *Xenopus* animal cap explants, but experiments point to Fgf signaling being permissive for mesoderm development rather than inductive (Kimmelman, 1987; LaBonne, 1995). In *Xenopus* and zebrafish embryos lacking Fgf signaling, the expression of pan-mesodermal genes such as *brachyury* is reduced and some mesodermal structures are not formed (Cao et al., 2004; Griffin, 1995; Schulte-Merker, 1995), but when Fgf signaling is chemically inhibited prior to mesoderm formation, axial mesoderm and the organizer are able to form (Fletcher and Harland, 2008). In the absence of Fgf, early mesoderm induction is intact, but expression of mesodermal genes is reduced during later stages of development indicating that Fgf is primarily involved with mesoderm patterning and/or maintenance (Schier, 2005).

Concurrent with mesoderm induction, the developing embryo establishes the dorsal-ventral (DV) axis and begins to pattern the nascent mesoderm along the DV axis (Figure 1.1C). DV patterning is achieved by competition between a broad ventralizing Bmp activity gradient and a restricted dorsalizing activity from the shield/dorsal organizer largely driven by the maternal β-catenin/Wnt signaling (Agathon, 2003; Ding et al., 2017; Kimelman, 2006; Schneider, 1996; Thisse and Thisse, 2015). The egg is patterned with an Animal-Vegetal axis around which it is radially symmetric. The initial radial polarity of the embryo is broken during fertilization; entry of the sperm through a micropyle at the animal pole causes maternally

deposited dorsal determinants to be transported to one side of the embryo, where they stimulate

the accumulation of β-catenin (Kelly, 2000; Schier, 2005; Schneider, 1996). This is

accomplished via transport along a polarized set of microtubules in the vegetal region of the

zebrafish embryo (Pelegri, 1998). The accumulation of β-catenin on one side of the embryo

induces a transcriptional program that specify formation of the gastrula or Spemann-Mangold

organizer that promotes dorsal gene expression, inhibits Bmp activity, and represses expression

of ventralizing factors (Agathon, 2003; Thisse and Thisse, 2015). Although Bmp signaling is

initially spread throughout the embryo, it later becomes restricted in the ventral mesoderm

(Langdon and Mullins, 2011; Tuazon and Mullins, 2015). The Bmp heterodimers induce

phosphorylation of Smad1/5, which accumulates in the nucleus and acts as a transcription factor

to drive expression of ventricle patterning genes. In turn, additional ventral determining factors

including *fgf* and *wnt8a*, which initially are expressed along the embryo circumference with

dorsally expressed Bozozok transcription factor and secreted Sqt, Cyc, and Bmp-antagonists,

including Chordin and Noggin, set up a gradient of ventral to dorsal identity, and will pattern the

nascent mesoderm along the DV axis (Leung, 2003; Thisse and Thisse, 2015).

It is important to note that during induction and DV patterning of the mesoderm, an

important change in gene expression is taking place in the developing embryo-maternal to

zygotic transition (Lee et al., 2014). For the first 2 hours of development, the mother deposited

the genes expressed in the embryos in the egg. Following zygotic activation of the genome, the

embryo begins to transcribe and translate its own genome. Some genes are required only

zygotically in the genome, others maternally, and a high percentage of the zebrafish genome is

required maternally and zygotically (Vesterlund, 2011). Mesoderm induction and patterning

requires both maternally and zygotically expressed genes, often requiring a maternally deposited

protein to repress the expression of a zygotically expressed gene, as in the case of B-catenin repressing *wnt8a* (Abrams and Mullins, 2009). Discerning the full role of a protein or gene in this process can require removing both maternal and zygotic function (Ciruna et al., 2002).

The arrangement of mesodermal progenitor fates along the DV axis is conserved from fish to mammals (Figure 1.2, adapted from (Schier, 2005) (Schier, 2005; Solnica-Krezel and Sepich, 2012; Tam, 1997). The dorsal organizer becomes the axial mesoderm and gives rise to the notochord and prechordal mesoderm. Flanking the axial mesoderm is the paraxial mesoderm, which gives rise to somites which go onto form the muscles and connective tissues. Laterally next to this is the intermediate mesoderm that gives rise to reproductive system and the kidney. Flanking that is the lateral plate mesoderm that will differentiate into the heart, body wall, blood, and other parts of the circulatory system (Dosch, 1997; Kimelman, 2006; Schier, 2005). The most ventrally located mesoderm will contribute to the tailbud and more posterior embryonic tissues in zebrafish, or to the extra embryonic mesoderm in the mouse. With the initial organization of the mesoderm (as well as endoderm and ectoderm) established, the embryo will begin specifying the organs and tissues that arise from each germ layer.

Shield Stage

6-Somite Stage

Axial Mesoderm
Paraxial Mesoderm
Intermediate Mesoderm
Anterior Lateral Plate Mesoderm
Ventral Mesoderm
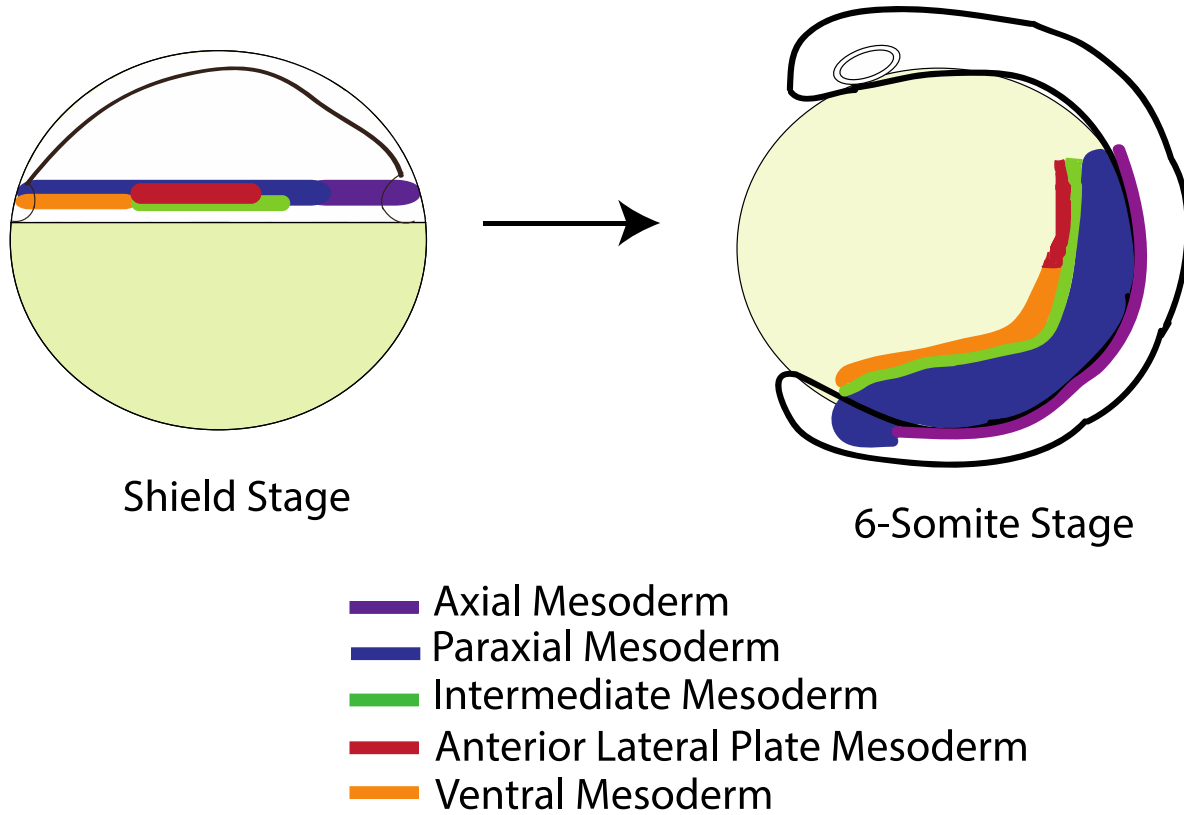
Figure 1.2 Mesoderm patterning in zebrafish

Organization of mesoderm sub-types is maintained in zebrafish embryos after gastrulation.

1.3 Anterior Lateral Plate Mesoderm Patterning

After mesoderm induction and broad DV patterning, the next step the embryo undertakes in forming the heart is generating cardiac competent mesoderm. Fate-mapping studies in chick, frog, zebrafish and mouse (Evans et al., 2010; Keegan et al., 2004) have demonstrated that the future cardiomyocytes and endocardium are located in bilateral regions of the anterior lateral plate mesoderm (ALPM), a subset of the described above lateral plate mesoderm, during gastrulation (Haack and Abdelilah-Seyfried, 2016). Many secreted factors coordinate to demarcate the ALPM region including Wnt, Bmp, Fgf, and retinoic acid (RA) (Begermann, 2001; Liu and Stainier, 2012). Bmps and Fgfs act as pro-cardiac signals, while Wnt and RA signaling primarily act to restrict the areas of cardiac-competent mesoderm (Bakkers, 2011; Brade et al., 2006; Reifers et al., 2000; Scott, 2012). The gradient of morphogens set-up by the ventral and dorsal patterning regions of the embryo will induce expression of cardiomyocyte transcription factors.

During gastrulation, the nascent cardiomyocytes will migrate from the margin in an organized manner that preserves the spatial distribution of the future ventricle and atrial cardiomyocytes established during DV patterning (Keegan et al., 2004). On bilateral sides of the embryo, the presumptive cardiomyocytes closest to the margin will internalize first subsequently followed by the further away levels (Keegan et al., 2004; Sepich, 2000). Directed mesoderm morphogenesis movements will move the nascent cardiomyocytes away from the margin towards to dorsal embryonic midline (Sepich, 2000; Solnica-Krezel and Sepich, 2012; Zeng et al., 2007). The cardiac competent mesoderm from both sides of the embryo will be arranged in bilateral stripes flanking the paraxial mesoderm. Once gastrulation movements have arranged the nascent cardiomyocytes, expression of cardiomyocyte specific genes will commence.

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At the beginning of somitogenesis, cardiac transcription factors are expressed in the

ALPM; these proteins will drive the specification of cardiomyocytes within the ALPM (Figure

1.1 D) (Bodmer, 1993; Ueyama et al., 2003). The first cardiac transcription factor to definitively

label the cardiac progenitor cells (CPCs) in both vertebrates and invertebrates is Nkx2.5, a highly

conserved homeobox domain protein required in heart development from fruit flies to humans

(Bakkers, 2011; Bodmer, 1993; Tanaka, 1999). As the ALPM begins to differentiate, genes

encoding cardiac transcriptions factors are expressed in the nascent cardiomyocytes of the

embryo including those encoding the zinc-finger proteins of the Gata family (*gata4*, *gata5*, and

gata6) (Evans, 1997), and others such as *mef2c*, *nkx2.7*, *tbx5* and *hand2* (Bruneau, 1999;

Durocher et al., 1997; Kyu-Ho, 1996; Yelon, 2000). The cardiac transcription factors delineate

the ALPM field from which cardiomyocytes emerge and initiate expression of the downstream

cardiac cascade.

These cardiac transcription factors occupy unique, yet partially overlapping niches in

shaping the heart (Bruneau, 2013). There is a vast array of cardiac transcription factors that are

required for heart development and fulfill tasks from differentiation of the cardiomyocytes to

cardiac chamber patterning (Roche et al., 2013). For example, the evolutionarily conserved

Nkx2.5 is required for the terminal differentiation of the myocardium. However, while Nkx2.5 is

required for heart formation in invertebrates, vertebrates still form a partial heart in the absence

of Nkx2.5 suggesting that other cardiac transcription factors can compensate for it (Bodmer,

1993; Roche et al., 2013). Hand2 regulates differentiation of the cardiac chambers and is

necessary for proper merging of the cardiac fields, while loss of Gata4 results in gross abnormal

heart morphology in mouse (Bruneau, 2013; Roche et al., 2013). The overlapping expression

patterns of cardiac transcription factors, their ability of some of them to form heterodimers, and

other experimental evidence point to their overlapping activity and co-regulation of the cardiac transcription factors by each other during heart development (Brown et al., 2004; Bruneau, 2013). In addition, chromatin immunoprecipitation studies indicate that Nkx2.5, Tbx5, Mef2c, and Gata4 bind one another and cooperatively activate each others' transcription (Brown et al., 2004; Bruneau, 2013; He, 2011; McCulley and Black, 2012).

The co-regulation and heterodimerization of cardiac transcription factors has implications in heart development and disease. Hypomorphic and heterozygous mutations in the human *NKX2.5* homolog can result in disease states (Balci and Akdemir, 2011; Bruneau, 2013; Jay, 2003; Lyons, 1995). Heterozygous mutations in *TBX5* result in defects in both heart and limb formation know as Holt-Oram Syndrome (Bruneau, 2013; Steimle and Moskowitz, 2017). The combinatorial and redundant functions of cardiac transcription factors make heart development robust while also requiring tight regulation of gene expression levels that can make the process vulnerable to defects.

1.4 Bilteral Heart Field Merging and Heart Looping

Once the cardiac transcription factors have differentiated the cardiomyocytes and endocardium, the bilateral heart fields must next merge through migration towards the embryonic midline (Figure 1.1 E) (Scott, 2012). In zebrafish, when the two heart fields merge, they form a structure known as the cardiac cone (Holtzman et al., 2007). The inner most cells of the cardiac fields are the endocardium, and they may help guide the cardiomyocytes to the midline (Holtzman et al., 2007). The medial most cardiomyocytes of the cone become the atrium of the heart and the surrounding cardiomyocytes form the ventricle. The cardiac cone then undergoes elongation and reorganization through a series of morphogenetic movements to shape

the primitive heart tube into its two chambers with the endocardium lining them (Glickman, 2002; Holtzman et al., 2007).

Merging of the first heart field from two bilateral regions is driven by migration of the cardiomyocytes atop the endoderm (Holtzman et al., 2007). Abnormal endoderm development and migration is observed in many zebrafish mutants that also manifest with cardia bifida (Reiter, 1999; Varner and Taber, 2012; Ye and Lin, 2013). Endoderm has both an instructive role in cardiomyocyte migration as well as providing a physical substrate over which the cardiomyocytes migrate (Varner and Taber, 2012; Ye and Lin, 2013). It is has been hypothesized that endoderm releases as yet unknown signaling molecules that guide the cardiomyocytes to the embryonic midline (Lin et al., 2005).

Once the cardiac tube is formed, it must undergo a series of morphogenetic movements to shape the cardiac chambers (Bakkers, 2011; Bakkers et al., 2009). The cardiac tube will extend right and then undergo a rotation that drives rightward looping of the heart. The rotation results from conformation changes in the cardiomyocytes of the atrium and ventricle (Haack and Abdelilah-Seyfried, 2016). The endocardium will form the atrioventricular (AV) valve that will separate the two chambers, and endocardial cells will balloon through proliferation to increase the size of the heart (Bakkers, 2011). By the end of this process, the heart will contain two visually distinct cardiac chambers that will have different genetic profiles and functions.

1.5 Expansion of the Cardiomyocyte Population

The embryonic heart forms from two genetically and spatially distinct populations of cells, the first and second heart fields (Buckingham et al., 2005; Guner-Ataman et al., 2013). The

first heart field consists of *nkx2.5* and *gata4* positive cardiomyocytes specified in the ALPM mesoderm during early somitogenesis; these cardiomyocytes will form the initial heart tube and ultimately contribute to the main chambers of the heart (de Pater et al., 2009; Kelly et al., 2014; Matrone et al., 2017). The first mechanism by which the heart tube will grow is by additional specification of cardiomyocytes from the undifferentiated ALPM surrounding the heart tube (Kelly et al., 2014). These cardiomyocytes comprise the second heart field and contribute mainly to the outflow tract of the heart (Buckingham et al., 2005; Mjaatvect, 2001). The second heart field is genetically distinct from the first heart field as its initial progenitors express *islet1* rather than *nkx2.5* like the first heart field (Cai, 2003).

After heart looping, heart growth is driven solely by proliferation of existing cardiomyocytes (Foglia and Poss, 2016). In zebrafish, cardiomyocyte proliferation is relatively high after heart tube formation with the heart gaining approximately 100 cardiomyocytes per day from 24 hpf through 96 hpf (Bennett et al., 2013; Matrone et al., 2015). As the heart matures, proliferation becomes reduced until it is nearly absent in the adult heart (Takeuchi et al., 2011).

As in other cells, cardiac cell proliferation is regulated through a collection of proteins called Cyclins (Bertoli et al., 2013; Hydbring et al., 2016; Ponnusamy et al., 2017). Cyclins derive their name from their cyclical expression during the proliferation; expression and protein levels of Cyclins rise during certain stages of the cell-cycle and fall as they are degraded (Lim and Kaldis, 2013). Cyclin proteins complex with Cyclin-dependent kinases at specific times in the cell-cycle and regulate progression through cell-cycle checkpoints and by phosphorylation of proteins and transcriptional regulation of genes (Lim and Kaldis, 2013). In vertebrates and invertebrates, *Cyclin D* is expressed highest during G1 of the cell cycle. *Cyclin E* is upregulated during the transition between G1 and S-phases. *Cyclin A* is expressed from S-phase through M-

phase, and *Cyclin B* is primarily expressed during M-phase (Bertoli et al., 2013). Regulation of Cyclins is key in the later post-mitotic state of the heart after birth in higher level vertebrates with specifically G/1 S-Phase *Cyclins* and G2/M-phase *Cyclins* have reduced expression levels.

Through studies in mouse and fish, a few of the cardiac transcription factors have been implicated in controlling cardiomyocyte proliferation (McCulley and Black, 2012). Depending on the stage, both Nkx2.5 and Gata4 can regulate the expression of *cyclin D2* (McCulley and Black, 2012; Rojas et al., 2008), Furthermore, Gata4 is a co-factor of Cyclin D2 during cardiogenesis (Yamak et al., 2014). The regulation of Cyclins by cardiac transcription factors may help explain how cardiomyocytes exit the cell-cycle after birth in mouse and human.

1.6 Cardiac Chamber Patterning

The heart tube undergoes a series of morphology changes that will shape the heart into discrete chambers with unique functions, geometries, and genetic signatures. In jawed fish, such as the zebrafish, the heart is comprised of two chambers: a single atrium and ventricle. In amphibians the heart has three chambers with two atria and a single ventricle. The mammalian and avian hearts contain four chambers with two atria and two ventricles.

Briefly, in zebrafish, heart looping occurs by the bending and twisting rightward of the linear heart tube. The two chambers become visually distinguishable when the heart tube expands by ballooning, and the chambers are separated by the constriction of the atrioventricular valve (Bakkers, 2011). Cell comprising the two chambers will have unique shapes and express distinct myosins, which are expressed differentially, even prior to heart field merging (Targoff et al., 2008). The ventricular cells will express *ventricle myosin heavy chain/myosin heavy chain 7* (*vmhc/myh7*) while atrial cells express *atria myosin heavy chain/myosin heavy chain 6*

(*amhc/myh6*) (Figure 1.1 F) (Bakkers, 2011). A third population of cardiac cells, the endocardium, lines the two chambers and is critical in forming the valves that separate the two chambers (Harris and Black, 2010).

Several of the cardiac transcription factors that initially help differentiate cardiomyocytes are repurposed during later heart development to pattern and maintain cardiac chamber identity (Bao, 1999; Bruneau, 1999; Bruneau et al., 2001; George et al., 2015; McCulley and Black, 2012; Stainier, 2001; Yutzey, 1994, 1995). Transcription factors Hey2, and Tbx5 repress atrial fate while Irx4 activates a ventricle specific genetic suite (Bruneau et al., 2001; McCulley and Black, 2012; Targoff et al., 2008). The cardiac transcription factors Nkx2.5 and its semi-redundant homolog, Nkx2.7, maintain ventricular identity in the zebrafish (Targoff et al., 2013; Targoff et al., 2008). Removal of both *nkx2.5* and *nkx2.7*, results in an almost complete loss of ventricular identity (Kyu-Ho, 1996; Targoff et al., 2013; Tu et al., 2009). Notably, loss-of-function mutations in cardiac genes often lead to a reduction in the expression levels of ventricle specific genes, indicating that atrial identity could be the default fate of cardiomyocytes (Stainier, 2001; Yutzey, 1994, 1995).

1.7 Epigenetic Regulation of Heart Development

Heart development requires that diverse processes from mesoderm patterning to cardiac chamber specification occur with precise spatiotemporal regulation. This in turn demands exquisite coordination of expression of hundreds of genes during early development. Questions still remain regarding how the embryo coordinates this process. Studies increasingly point to chromatin modifications playing essential roles in regulating the expression of genes during heart development to coordinate spatiotemporal expression necessary for building the heart.

Transcription is strongly influenced by modifications of DNA associated proteins. DNA in the nucleus is wrapped around histones containing nucleosomes that both compact the DNA as well as regulating the ability of DNA to be transcribed. The addition of covalent histone modifications such as H3K4me1, H3K4me3, H3K27me1, H3K27me3, H3K9ac, and H3K27ac open the chromatin structure allowing access of the transcriptional machinery and are considered "activating"(Harr et al., 2016). Chromatin modifications that inhibit transcription of the DNA include H3K9me2, H3K9me3, H3K27me2, and H3K27me3 are considered "repressive". Methyltransferase proteins place and remove methyl marks on histones while histone acetyltransferase (HATs) place acetyl marks and histone deacetyltransferases (HDACs) remove acetyl marks (Bannister and Kouzarides, 2011).

In addition to proteins that covalently alter histone modifications, a suite of proteins called nucleosome remodelers regulate gene expression by moving or assembling nucleosome, eight histone protein cores around which DNA is wrapped, to either make genes more or less accessible to transcription and replication machinery (Becker and Workman, 2013). The four major sub-types of nucleosome remodelers implicated in heart development are SWI/SNF, ISWI, CHD, and INO80 (Hota and Bruneau, 2016). Tissue-specific isoforms of nucleosome remodelers enable the complexes to initiate expression of a suite of genes during heart development (Hota and Bruneau, 2016).

Histone modifiers and nucleosome remodelers function in mesoderm development and ALPM patterning. Activin/Smad2 may modulate mesoderm development by reducing H3K27me3 levels, as it does in direct differentiation of embryonic stem cells into mesendoderm (Wang et al., 2017). Mesodermal genes are poised for expression through co-marking with repressive and activating histone modifications. The resolution of these bivalent histone marks, is

a key step in mesoderm induction (Barrero et al., 2010). The mesodermal fate specification and patterning genes *Brachyury, Gata4, Gata5, Nkx2.5, Tbx5*, and *Mef2C* contain bivalent histone modifications during early development (Barrero et al., 2010). Histone modifiers act to regulate gene expression and ready the genome for rapid activation towards expression of genes promoting mesoderm fate.

The cardiac lineage of mesoderm appears particularly sensitive to epigenetic regulation. Many chromatin remodelers, methyltransferases, and acetyltransferases are expressed in the heart during development, and they provide spatiotemporal attenuation of gene expression that is essential for heart formation (Chang and Bruneau, 2012; Han et al., 2011). Mutating or knocking-down a range of nucleosome remodelers including Baf60c sub-unit of the SWI/SNF complex result in abnormal heart formation such as reduced expansion of the second heart field (Lickert et al., 2004). Cardiac transcription factors control the expression of some chromatin modifiers. For example, Nkx2.5 inhibits the expression of Histone demethylase *Jarid2* during outflow tract development (Barth et al., 2010). Cardiac transcription factors appear to be co-dependent on histone modifiers and epigenetic markers both being regulated by them and acting with them to regulate expression of their target genes (Liu et al., 2009; Paige et al., 2012; Schlesinger et al., 2011).

Chromatin factors modulate other processes necessary for cardiogenesis, notably, cardiomyocyte proliferation. There are several instances of cell proliferation being influenced epigenetically. Histone demethylase *Jarid2* represses cardiomyocyte proliferation by removing methylation of the *Cyclin D* promoter (Jung et al., 2005; Nakajima et al., 2011). Furthermore, HDACs are associated with the promoter regions of cell cycle genes during development (Oyama et al., 2014). Together, these examples and others suggest that epigenetic modifiers

modulate the cell cycle during development as well as silencing proliferation post cardiomyocyte differentiation (Oyama et al., 2014) .

1.8 Gon4l

Gon4l is a putative chromatin remodeler (Lim et al., 2009; Liu et al., 2007b; Lu et al., 2011; Lu et al., 2010). This evolutionarily protein is comprised of more than 2,000 amino acids and consists of several domains (Figure 1.3). Similarity to other epigenetic modifiers hints at a function as a chromatin modifier (Lu et al., 2011). These domains include a YY1 binding domain, paired amphipathic domains, and a SANT-domain (Liu et al., 2007b; Lu et al., 2010). SANT domains are a 50-amino acid motif commonly found in chromatin modifying proteins and allow interaction with the lysine-containing histone tails (Boyer, 2004). Paired amphipathic domains function as protein-protein interaction domains for transcriptional repressors (Le Guezennec et al., 2006). Such paired amphipathic domains are found in the Sin3a transcriptional repressor protein (Le Guezennec et al., 2006). Finally, the YY1 binding domain in Gon4l shares homology with the primate specific YY1AP (Liu et al., 2007b; Wang et al., 2004). YY1AP binds the transcription factor YY1, which has both activating and repressing roles, and enhances the transcriptional activating function of YY1 (Wang et al., 2004). YY1 is notable as a transcription factor with diverse roles in development, including links to heart development, as well as the capability to both activate and repress genes expression depending on the context (Beketaev, 2015; Gordon et al., 2006; Gregoire et al., 2013; Nan and Huang, 2009).

Gon4l is an evolutionarily conserved gene among animals and shares domains with genes found in land plants (Brownfield et al., 2009) vertebrates and invertebrates (Friedman et al., 2000; Liu et al., 2007b). In both plants and animals, Gon4l and its homologues are required for

proper cell-cycle regulation (Barr et al., 2017; Brownfield et al., 2009; Liu et al., 2007b).

However, in animals, the protein has likely evolved additional functions.

1.8.1 *gon-4* in *C. elegans*

The *gonad-4* (*gon-4)* gene was discovered in a *C. elegans* screen for mutations in gonad development (Friedman et al., 2000). While overall animal morphology is grossly unaffected, the *gon-4* mutant worms fail to differentiate their germline, and neither the males nor the hermaphrodites produce progeny. The severity of the phenotype is highly variable with males containing as few as five undifferentiated germ cells to other males having germ line containing hundreds of undifferentiated germ cells. Likewise, the hermaphrodites exhibit a range of phenotypes including vulva-less, protruding vulva, and multiple vulvae.

Defects in germline differentiation in *gon-4* mutant *C. elegans* were traced to a failure of somatic gonad development (Friedman et al., 2000). Dramatic delays were observed in the initial divisions of the somatic germline precursors at the Z1/Z4 stage, but later steps of gonadogenesis proceed normally. Other cell divisions are normal in the developing worm. Experiments in *C. elegans* indicated that the Gon-4 protein localizes to the nucleus and likely acts cell-autonomously during gonad development.
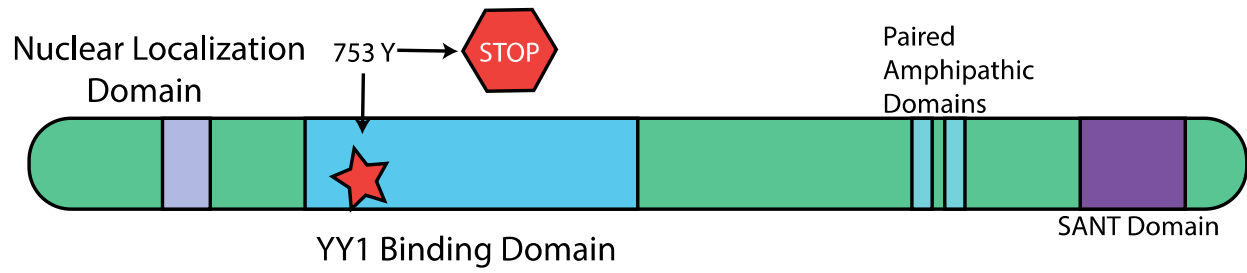
Figure 1.3 Gon4l protein

Gon4l contains a nuclear localization domain, YY1-binding domain, paired amphipathic domains, and a SANT domain. The *udu^{vu66}* creates a premature stop at the 753 amino acid.

1.8.2 *gon4l/udu* in Zebrafish

The first mutant for the zebrafish homolog of *gon-4*, known as *gonad-4-like* (*gon4l*) or *ugly duckling* (*udu*), was identified as a tail morphogenesis mutant in the large-scale forward genetic screen in Tubingen during the early 1990s (Hammerschimidt et al., 1996). In contrast to the overtly normal morphology of the *gon-4* mutant in *C. elegans*, *udu* mutant zebrafish have pronounced morphological defects including a shortened body axis, severe heart edema, somite formation defects, smaller head, and misshapen tail (Hammerschimidt et al., 1996; Liu et al., 2007b; Williams et al., 2018).

Identification of additional zebrafish *udu* mutant alleles revealed defects in hematopoiesis, specifically a failure to differentiate erythroid cells (Liu et al., 2007b). Both increased cell death and decreased proliferation were noted in the *udu* mutant zebrafish embryos, but the defects in hematopoesis appear to be largely due to increased cell death (Liu et al., 2007b). *udu* mutant zebrafish embryos injected with transcription blocking morpholino oligos against *p53,* an essential component of the cell death pathway, exhibit a partial restoration of blood development (Liu et al., 2007b). Lim et al. found that *p53* upregulation in *udu* mutant zebrafish embryos is due to activation of the Atm-Chk2 damage response pathway (Lim et al., 2009). Apoptosis could also be reduced in the *udu* mutant zebrafish embryos by inhibiting the Atm-Chk2 pathway via injection of morpholino oligos against *chk2*. Together, these results indicate that Gon4l promotes cell survival during hematopoiesis and enables terminal differentiation of blood cells.

In examining hematopoietic defects in *udu* mutant embryos, it was noted that the blood stem-cells displayed a decreased level of proliferation (Lim et al., 2009). Intriguingly, the researchers identified minichromosome maintenance complex (MCM) proteins MCM3 and

MCM4 as binding partners of Gon4l. MCM proteins are essential for DNA replication and bind to the DNA during G1/S phase of the cell-cycle and become dislodged as replicated proceeds (Forsburg, 2004). MCM3 is believed to be phosphorylated by Cyclin E, which regulates initiation of S-phase of the cell cycle (Li et al., 2011). *udu* mutant zebrafish erythropoetic (blood cells) accumulate in the G2/M phase of the cell cycle, indicating a blockage in the cell-cycle, but it has not be demonstrated if overcoming this blockage can restore blood development (Lim et al., 2009). Despite the defects in blood development and somites, it has not be tested in Gon4l has functions in other mesoderm tissues including the heart.

1.8.3 Gon4l in Mouse

Hypomorphic mutations in the murine *Gon4l* homolog result in embryos with hematopoietic defects confirming an evolutionarily conserved function for the gene in blood development (Lu et al., 2010). The *Gon4l*-deficient mouse embryos are viable, although by 6 months of age, 25% have developed spontaneous salivary tumors (Simons et al., 2013). The *Gon4l* mutant mouse fail to differentiate B-cells and only develop T-cells, hence the name *Justy* for this hypomorphic allele (Lu et al., 2010). Unlike in zebrafish embryos, suppressing cell death by blocking *TP53* expression did not restore blood development in the Justy mouse mutants (Barr et al., 2017; Liu et al., 2007b). However, similar to zebrafish, BrdU labeling of mouse pre-pro-B cells revealed that a reduction of proliferation in the *Gon4l* mutant cells relative to WT with fewer cells in S-phase and G2 of the cell-cycle (Barr et al., 2017; Lim et al., 2009).

The expression of many cyclins, which are the major regulators of the cell cycle, was downregulated in *Gon4l* mutant B-cell progenitors suggesting a potential mechanism for the reduced number of erythroid cells (Barr et al., 2017). Notably, *Ccnd1, Ccnd2, Ccnd3, Ccna2, Ccnb1, Cnnb2*, and *Ccnf* all have reduced expression levels (Barr et al., 2017). Enforced

expression of the G1/S-phase regulators *Cnnd3* and *Ccne1* in cultured cells significantly promotes B-cell development in *Gon4l* deficient cells. Interestingly, enforced expression of *Ccna2* and Cnnb1 has no effect on B-cell development in Gon4l-deficient cells. This result indicated that overcoming the mitotic block was sufficient to restore B-cell development but that Gon4l is not required to induce the B-cell differentiation process (Barr et al., 2017).

Gon4l has no DNA binding domains, implying that interactions between Gon4l and DNA must be mediated by binding partners (Lu et al., 2011). Co-sedimentation studies likewise found that the majority of Gon4l protein is found to stably interact with other proteins (Lu et al., 2011). The protein structure of Gon4l contains a region that is 78% similar to YY1 associated protein (YY1AP), a primate-specific partial duplication of Gon4l (Lu et al., 2011). YY1AP, as the name suggests is able to bind the transcription factor YY1 (Wang et al., 2004). Co-immunoprecipitation (Co-IP) revealed that Gon4l binds YY1 in the M12 cells, and that the interaction requires the YY1AP homology domain of Gon4l (Lu et al., 2011). Additional binding partners identified in Co-IP studies were the histone deactylase HDAC1 and transcriptional regulator Sin3a, which were shown to form a complex with Gon4l and YY1. This Gon4l, Sin3a, HDAC1, and Yy1 complex represses gene expression (Lu et al., 2011).

1.9 Links Between Gon4l and Heart Development

The interaction between Gon4l and YY1 is notable from the perspective of mesoderm and heart development. YY1 is a ubiquitously expressed zinc-finger transcription factor and member of the Polycomb Repressive Complex with multiple roles in development, cancer, differentiation, and proliferation (Aoyama et al., 2010; Atchison et al., 2003; Calame and Atchison, 2007; Gordon et al., 2006; Lee et al., 1995; Liu et al., 2007a; Lu et al., 2011; Nan and

Huang, 2009; Shi et al., 1991; Yao et al., 2001). YY1 protein contains DNA-binding domains

and has been extensively demonstrated to have both activating and repressing roles (Gordon et

al., 2006). When complexed with E1A, YY1 induces activation of transcription, but without

E1A, YY1 is known to act as a transcriptional repressor (Chang 1989, Shi, 1997).

In 2013, Gregoire et al. identified YY1 as a key transcriptional regulator of cardiac

differentiation. Loss of YY1 results in a failure of Natural Killer ESCs to differentiate into

cardiomyocytes. Similarly, YY1 loss-of-function mice are embryonic lethal and fail to specify

the cardiac lineage (Gregoire et al., 2013). The critical role of YY1 in heart development might

be attributed to the enrichment of YY1 at the enhancer region of the essential cardiac

transcription factor Nkx2.5 (Gregoire et al., 2013). This binding and activation of *Nkx2.5* by

YY1 is partially mediated through interactions with cardiac transcription factor Gata4. YY1 and

Gata-4 co-occupy regions at the *Nkx2.5* enhancer and can be pulled down together through a Co-

IP. In addition to its regulation of *Nkx2.5* expression, YY1 also binds at the enhancer regions of a

suite of developmentally important cardiac genes including *Mef2c, Pitx2, Tbx3,* and *Tbx5*

(Gregoire et al., 2013). Further analysis of the function of YY1 in cardiac development revealed

regulation of downstream cardiac genes such as *SRF, Mesp1, eHand, Cited* and *Dll1* suggesting

that YY1 sits atop a large cascade of cardiac genes (Beketaev, 2015).

Despite the evidence demonstrating that Gon4l complexes with the required cardiac

transcription factor YY1 and the broad disruption of mesoderm derived tissues in Gon4l mutant

animals including blood and somites, little research has been conducted into the roles of Gon4l in

mesoderm differentiation and thus far nothing has broached its potential roles in heart

development. This thesis project addresses both.

1.10 Objectives, Results, and Significance of Thesis

Gon4l is a highly conserved protein with defined functions in the development of mesodermal tissues, cell death, and cell-cycle regulation. Despite these known functions, the role of Gon4l in heart development remains poorly understood. This work defines distinct roles for maternal and zygotic expression of Gon4l in heart development. We demonstrate that Gon4l is required maternally to pattern the mesoderm and zygotically to regulate ventricular identity and cardiomyocyte proliferation. These investigates provide insights into the complex regulation underlying heart development and how the chromatin factor Gon4l is involved in regulating these processes.

Firstly, this study implicates maternal Gon4l function in mesoderm patterning. Previous work on Gon4l in zebrafish was carried out using only zygotic loss of function embryos (Lim et al., 2009; Liu et al., 2007b). Here in Chapter 2 and in a recent publication Williams, et al., 2018 we elucidate a hereunto known function for Gon4l in mesoderm patterning and ALPM specification. Through RNA-seq we demonstrate that loss of Gon4l function results in differential expression of 5-11% of the genome during development with mesoderm genes being especially affected.

Secondly, this study elucidates novel role for zygotic Gon4l function in heart development. *udu* mutant embryos have smaller hearts with reduced ventricular identity. We demonstrate that unlike in hematopoiesis, blocking apoptosis is not sufficient to restore heart development in udu mutant embryos. We identify reduced proliferation in udu mutant cardiomyocytes and cardiomyocyte-specific differential expression of the cyclins *cnne2* and *ccnd3*. We establish a role for Gon4l in maintaining ventricular identity of cardiomyocytes.

Through genetic interaction studies we establish that *udu* and *nkx2.5* genetically interact in maintaining ventricular identity in heart development.

This work investigates the roles of Gon4l in heart development in zebrafish, providing critical insight into a novel chromatin factor in heart development. Heart development necessitates careful spatiotemporal regulation of multiple processes from mesoderm induction through cardiomyocyte proliferation. This studies shed light onto how Gon4l regulates many of the complex genetic pathways underlying heart development and provides a new genetic target for future studies to better understand the developmental origins CHDs.

Chapter 2: Chromatin factor Gon4l aides germ layer patterning

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2.1 Abstract

One of the first tasks of embryogenesis is to induce formation of the three germ layers: endoderm, mesoderm, and ectoderm. Chromatin modifications play an important role in formation and patterning of the germ layers, but many of the genes involve remain unknown. Here we define a novel role of the chromatin factor Gon4l, encoded by *ugly duckling* (*udu*), in germ layer development with a focus on its functions in mesoderm patterning and specification of mesoderm derived tissues. We demonstrate that maternal and zygotic loss of Gon4l perturbs germ layer induction. Furthermore, complete loss of Gon4l function results in broad misreuglation of mesoderm tissues from the initial dorsal-ventral patterning of the embryo to the development of mesoderm derived organs. Cell autonomy transplantation experiments reveal that Gon4l is required both cell-autonomously and non-cell-autonomously in heart development. Finally, motif analysis of Gon4l associated sites reveals that Gon4l has motifs in common with transcription factors that act in segment specification and heart development.

2.2 Introduction

During gastrulation, the differentiation of totipotent cells begins with the specification of the three germ layers: endoderm, mesoderm, and ectoderm. Nodal ligands secreted from the yolk syncytial layer and the marginal blastomeres of the zebrafish blastula are the main inducer of germ layer specification (Conlon, 1994; Mizuno, 1999; Ober and Schulte-Merker, 1999; Shen, 2007; Thisse and Thisse, 2015). In the blastoderm shaped like an inverted cup residing on the

yolk syncytial cell, the future endoderm resides closest to yolk, followed by a broader band of mesoderm while ectoderm will be comprised of the animal most cells. The germ layers give rise to defined tissues (Williams and Solnica-Krezel, 2017). The ectoderm becomes the epidermis and neural tissues. The endoderm differentiates into the guts and its derivatives. Mesoderm derivatives include blood, somites, kidney, and heart, among many other tissues.

Dorsal and ventral organizing signals pattern the mesoderm into its sub-types. The dorsal organizer secretes *wnt* and *bmp* antagonists that inhibit action of morphogens expressed from the ventral side of the embryo and establishes a gradient of genes expression levels that will pattern the emerging mesoderm into its sub-types (Feldman, 1998; Kelly, 2000; Thisse and Thisse, 2015). Prior to gastrulation, the nascent mesoderm is organized with the future sub-types residing in loosely defined regions of the margin (Schier, 2005). From dorsal to ventral the mesoderm sub-types are: axial, paraxial, intermediate, lateral plate, and ventral. Axial mesoderm consists of the dorsal organizer and will form the notochord. Paraxial mesoderm differentiates into somites. The intermediate mesoderm will become the kidney and gonads. Anterior lateral plate mesoderm will give rise to the heart and blood vessels, while the ventral lateral plate mesoderm will give rise to the blood.

Epigenetic regulation provides a potential mechanism to ensure proper spatiotemporal expression of morphogens that can specify and pattern the germ layers, including mesoderm. Increasingly studies performed in model organisms are identifying roles for epigenetic modifiers in modulating gene expression and priming genes to facilitate rapid specification of the mesoderm during gastrulation. Essential mesoderm genes, including *ntl*, contain bivalent chromatin domains during early differentiation suggesting that epigenetic modifiers may help poise the genome for rapid differentiation of tissues during embryogenesis (Golob et al., 2008).

Epigenetic modifiers have been shown to modulate expression of the transcription factors that regulate the genetic cascade that controls specification of the paraxial, intermediate, lateral plate mesodermal territories from which subsequently differentiation of the somites, kidney, blood, and heart with arise (Chang and Bruneau, 2012; Delgado-Olguin et al., 2006; Harada et al., 2017; Hota and Bruneau, 2016; Hu, 2016; Ranghini and Dressler, 2016).

Here we propose a role for the chromatin factor, Gon4l, encoded by the zebrafish gene *ugly duckling* (*udu*), in facilitating germ layer and mesoderm patterning in the zebrafish embryo (Hammerschimidt et al., 1996; Liu et al., 2007b). In zebrafish, removal of only zygotic function of *udu* (Z*udu*) results in defects in somite and blood formation (Lim et al., 2009; Liu et al., 2007b). A hypomorphic allele of Gon4l in mouse demonstrates a conserved role in hematopoiesis (Lu et al., 2010). In a previous publication, our lab showed that removal of maternal and zygotic function of *udu* in zebrafish caused defects in notochord boundary formation, but other roles in mesoderm development were not fully explored in the MZ*udu* embryos (Williams et al., 2018). Here we show that Gon4l function is essential for germ layer patterning mesoderm with a special focus on mesoderm and its derivatives. While initial specification of the germ layers, including mesoderm is normal in MZ*udu* mutant embryos, the subsequent process of patterning of the mesoderm is disrupted, resulting in drastic abnormalities or loss of most mesoderm-derived tissues.

2.3 Material and Methods

Zebrafish strains and embryo staging

Adult zebrafish were raised and maintained according to established methods in compliance with standards established by the Washington University Institutional Animal Care and Use

Committee. Fish were fed with rotifers during larval stages and rotifers and dry food during adulthood. Embryos were obtained from natural matings, maintained at 28.5° C, and staged according to Kimmel's stages (Kimmel, 1995). All WT studies were carried out in AB*or AB*/Tübingen backgrounds. Additional lines used include *udu^{vu66}* and Tg(*myl7*:e*GFP*) (Huang et al., 2003; Williams et al., 2018).

Generation of maternal-zygotic (MZ) *udu* embryos

Germline replaced fish harboring *udu^{vu66/vu66}* germ cells, were generated by the method described in (Ciruna et al., 2002). Briefly, donor embryos from *udu^{+/vu66}* intercrosses or females and males with *udu^{vu66/vu66}* germline were injected with synthetic *GFP-nos1-3'UTR* RNA encoding Green Fluorescent Protein to fluorescently label the germline, and WT host embryos were injected with a morpholino oligonucleotide targeting *dead end1* (MO1-*dnd1*) to eliminate host germ cells. Between 50-100 cells were transplanted from the embryonic margin of donor blastulae to the embryonic margin of hosts at sphere stage (4 hpf). Both hosts and donors were cultured in agarose-coated plates in 0.3X Danieau treated with 100μg/mL penicillin streptomycin. Host embryos were screened for GFP+ germ cells at 36-48 hpf, and the genotype of corresponding donors was determined by phenotype. All putative *udu^{vu66/vu66}* germline hosts were raised to adulthood and confirmed by crossing to *udu^{vu66/+}* animals prior to use in experiments. More than 25 females and 25 males with a replaced germline were generated and used during the course of these studies.

Whole mount *in situ* hybridization (WISH)

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS). Embryos older than 24 hpf were bleached after fixation to remove pigment solution containing 3% H_2O_2 and 0.5% KOH prior to carrying out the WISH (Thisse and Thisse, 2008). WISH was performed as previously described using 65% formamide solution and 70°C hybridization temperature for the following probes: *myosin light chain 7* (*myl7)* (Yelon et al., 1999)*, myosin heavy chain 6 (myh6)* (Yelon et al., 1999) and *myosin heavy chain 7 (myh7)* (*Yelon, 2000)*, *nkx2.5* (Chen and Fishman, 1996), *nkx2.7* (Reifers et al., 2000), *hand2 (Yelon, 2000), gata4 (Serbedzija G., 1998), gata5* (Pack, 1996), and *bmp4* (Chen, 1997). Embryos were imaged using a Nikon AZ100 dissecting microscope.

Cell Autonomy Transplantations

Cell transplantations were performed as previously described (Ho, 1990; Thomas et al., 2008). In summary, donor embryos carrying the Tg(*myl7:eGFP*) transgene were injected at one-cell stage with 1μL of 5% tetramethylrhodamine (rhodamine) dextran (10,000 MW). At shield stage, 40-50 cells were transplanted from the margin of the host to the margin of the donor. Embryos were maintained in 0.3X Danieau with penicillin streptomycin until they were analyzed by compound microscopy at 48 hpf to assess contribution of donor cells to the heart based on Tg(*myl7:eGFP*) expression. Images were taken using a using a Zeiss Imager Z1 outfitted with RFP and GFP filters. 50 1μm Z-slices were taken per embryo and channels were overlaid and analyzed using FIJI.

Cardiomyocyte Counting

Hearts were manually dissected from embryos carrying a *myl7:eGFP* transgene using 29G

needles and briefly fixed in 4% PFA (Yang and Xu, 2012). Hearts were washed three times with

phosphate buffered saline containing 0.1% tween (PBST). 2-(4-amidinophenyl)-1H -indole-6-

carboxamidine (DAPI) was added for three minutes to label the nuclei. Then hearts were washed

three times with PBT and mounted in 2% methylcellulose for imaging on a coverslip. Hearts

were imaged using at 20X objective using an Olympus IX81 inverted spinning disc confocal

microscope. Cardiomyocytes were manually counted in FIJI.

Cell Tracking and Analysis

Tg(*myl7:eGFP*) embryos at the 16-somite stage, according to Kimmel's stages, were mounted

dorsal-down in 1% low melt agarose (Holtzman et al., 2007; Kimmel, 1995). Embryos were

imaged using an Olympus IX81 inverted spinning disc confocal microscope with 40-50 slices

of 0.5μm. 3 hour time-lapse movies were created and analyzed using FIJI. 15-20 cardiomyocytes

were followed from each WT embryo. All possible cardiomyocytes were followed in MZ*udu*

mutant embryos. Data was calculated in Python.

RNA-sequencing

RNA was isolated from 50-100 WT or MZ*udu* embryos per sample at shield and 15-somite stage

according to instructions for the Dynabeads mRNA direct kit (Ambion). Two biological

replicates per genotype were split into two technical replicates each to yield a total of four

independently prepared libraries. Libraries were prepared according to instructions for the

Epicentre ScriptSeq v2 RNA-seq Library preparation kit (Illumina). RNA was enzymatically

fragmented prior to cDNA synthesis. Next, the cDNA was 3' tagged and purified using

Agencourt AMPure beads. Sequencing indexes were added to cDNA during PCR amplification. Purified indexed libraries were submitted to the Washington University Genome Technology Access Center (GTAC) for sequencing using an Illumina HiSeq 2500 to obtain single-ended 50bp reads. Using STAR (2.4.2a) with default parameters raw reads were mapped to the GRCz10 reference genome. FeatureCounts (v1.4.6) from the Subread package was used to quantify uniquely mapped reads (phred score$\geq$10) to gene features based on the Ensembl annotations (v83). Differentially expressed genes were identified by using DESeq2 in the negative binomial distribution model with a cutoff of adjusted p- 5 value$\leq$0.01 and fold-change$\geq$2.0. Heatmaps were built using the heatmaps2 package in R, and other plots were built using the ggplot2 package in R.

Statistical analyses

Statistical tests were conducted using GraphPad Prism v.4, R, and Microsoft Excel.

2.4 Results

2.4.1 Loss of maternal and zygotic *udu* expression affects expression of dorsal-ventral patterning genes during early embryogenesis
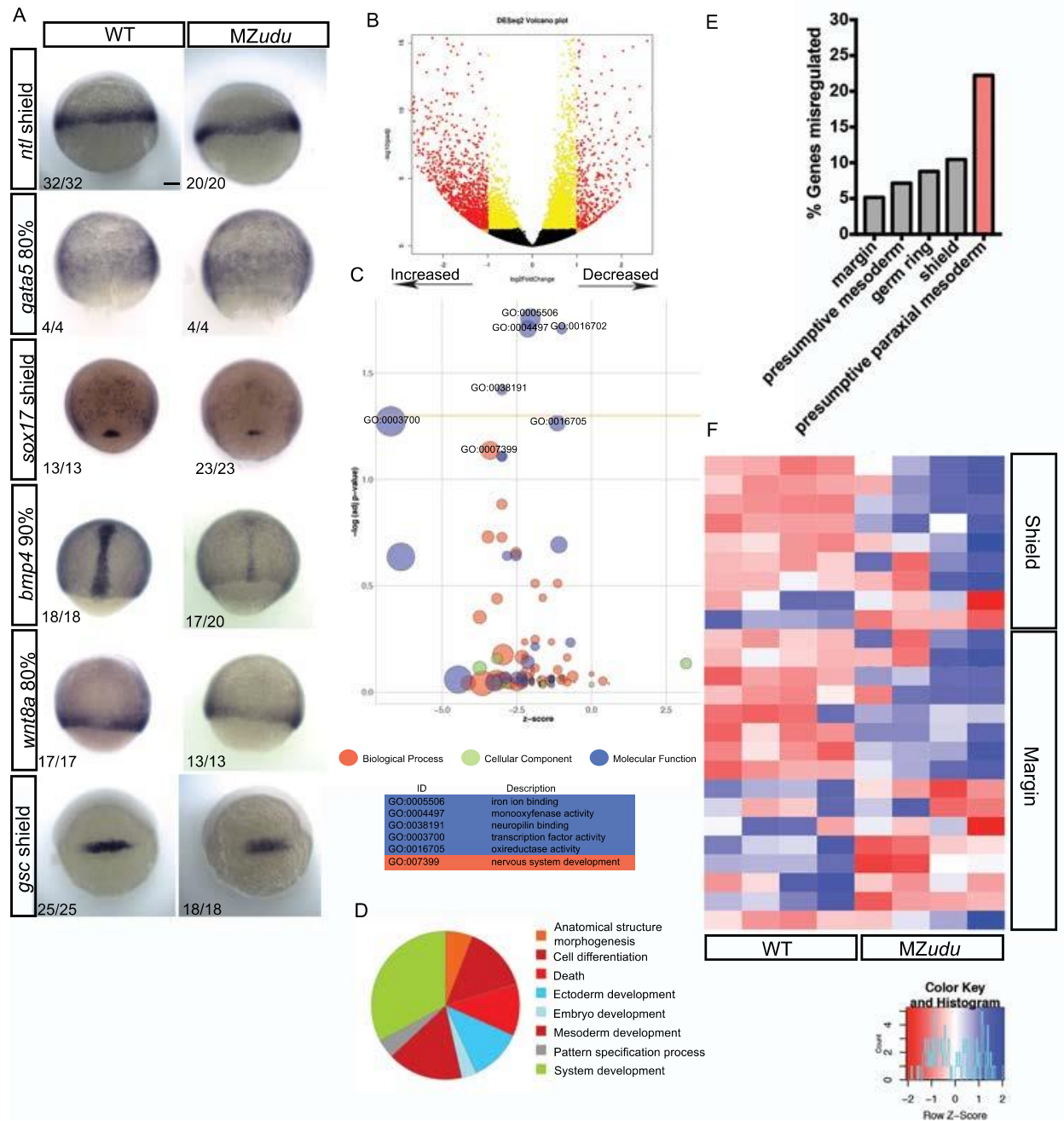
Figure 2.1 MZ*udu* embryos form germ layers but aberrantly express mesoderm and endoderm patterning genes

(A) WISH markers for nascent mesoderm (*ntl*), mesendoderm (*gata5*), and endoderm (*sox17*) during early gastrulation stages. Dorsal view of ventral (*bmp4* and *wnt8a*) and dorsal (*gsc*) WISH markers at early gastrulation stages. (B) Plot of gene expression changes in MZ*udu* embryos compared to WT at shield stage. Red dots represent genes with significantly different expression levels than WT (p≤0.05 and ≥2 fold change). Plot showing gene ontology enriched terms among the genes differentially expressed in shield stage MZ*udu* embryos. Categories shown are biological process (red), cellular component (green), and molecular function (blue). Bubble size represents number of genes per category. Log p-value plotted along Y-axis and Z-score plotted along X-axis. (D) Plot of "Developmental Process" GO-term and percentage of genes within each sub category. (E) Percent of genes associated with GO-terms associated with mesoderm induction that are differentially expressed in shield-stage RNA-seq. (F) Heat map of genes associated with the margin and shield anatomical structures upregulated (blue) and downregulated (red) at shield stage. Scale bar represent 50μm.

Zebrafish embryos lacking only zygotic expression of *udu* have defects in the formation of mesoderm-derived tissues including somites, blood, and heart (Lim et al., 2009; Liu et al., 2007b). Our lab has previously reported that full removal of Gon4l function through generation of embryos lacking both maternal and zygotic (MZ) contribution of Gon4l via germline replacement exhibit defects in axial extension, axial mesoderm cell polarity and notochord boundary formation (Williams et al., 2018). We wanted to understand the earliest perturbation in mesoderm development that arise in the MZ*udu* mutant embryos and if the defects in mesoderm tissues extend beyond the notochord and somites.

The nascent mesoderm is induced in zebrafish embryos soon after the onset of zygotic expression, such that just prior to the start of gastrulation mesodermal and endodermal precursors are intermixed at the blastoderm margin in a tissue layer known as mesendoderm. We sought to characterize mesoderm from the induction through dorsal-ventral (DV) and anteroposterior patterning phases. Through WISH we analyzed the expression of the nascent mesoderm and endoderm. We found that at the shield stage the presumptive mesoderm as marked by *ntl* exhibited comparable expression patterns in MZ*udu* (n=20/20) mutant embryos as in WT (n=32/32) (Figure 2.1 A). *gata5*, which marks mesendoderm, also had normal expression patterns at 80% epiboly stage in MZ*udu* (n=4/4) mutant embryos relative to WT (n=4/4). However, at the same 80% epiboly stage, *sox17*, an endoderm marker, presented a slightly reduced expression domain in MZ*udu* mutant gastrulae (n=13/13) relative to WT (n=23/23). These results indicate that MZ*udu* mutant embryos are able to induce mesoderm and endoderm, but germ layer formation is mildly perturbed.

Having established that mesoderm and endoderm germ layers are induced in MZ*udu*$^{-/-}$ embryos, we wanted to interrogate if the expression domains of genes that pattern the nascent mesoderm along the dorsal-ventral (DV) axis are disrupted. b-catenin and then Bone morphogenetic protein (BMP)-dependent DV patterning breaks the initial radial symmetry of the embryo and provides the initial instructions that will drive the mesoderm towards its later component sub-types (Langdon and Mullins, 2011; Solnica-Krezel and Sepich, 2012; Thisse and Thisse, 2015). We employed WISH labeling for *gsc* to examine formation of the shield, equivalent of the dorsal gastrula organizer in zebrafish in the MZ*udu* mutant and test if the embryos are establishing the dorsal organizer. We observed that all of the WT (n=25/25) and MZ*udu* (n=18/18) mutant embryos expressed *gsc*. We then observed the expression patterns of the *bmp4* and *wnt8a* genes encoding secreted factors that promote ventral and posterior fates and found that both displayed reduced domains in MZ*udu* embryos (n=17/20, n=13/13) relative to WT embryos (n=17/17, n=18/18) (Figure 2.1 A). Together, these results indicate a disruption in genes expressed early in mesoderm patterning.

As a chromatin factor, Gon4l could be affecting the expression of many genes during early gastrulation. We wondered if these deviations in the expression domains of mesoderm related genes could reflect wider changes in gene expression during early gastrulation. To provide a more comprehensive view of how loss of Gon4l affects gene expression we performed RNA-seq on shield-stage WT and MZ*udu* embryos. Analysis of relative transcription levels revealed that approximately 6% (1684 genes) of the zebrafish genome was differentially expressed in shield-stage MZ*udu*$^{-/-}$ embryos (p<0.05, >2-fold). Approximately 75% of the differentially expressed genes were upregulated at this stage (Figure 2.1 B).

Gene ontology (GO) term analysis of the genes differentially expressed at shield-stage in MZ*udu* embryos enriched for cellular component and molecular function related to metal ion binding and transcription factor activity (Figure 2.1 C) (Huang da et al., 2009a, b). We were particularly interested in developmental process with differentially expressed genes in MZ*udu*$^{-/-}$ embryos. GO term analysis of the differentially expressed genes also identified enrichment for functional annotations for terms related to mesoderm and ectoderm development (Figure 2.1 D) (Doerks et al., 2002).

We used Zebrafish Expression Ontology of Gene Sets (ZEOGs) (Prykhozhij, 2013), a zebrafish-specific annotation tool, to compile lists tissue-specific genes associated with mesoderm in the shield stage embryo, including the margin, presumptive mesoderm, germ ring, shield, and presumptive paraxial mesoderm. These genes lists were compared to the genes differentially expressed in RNA-seq datasets from shield stage embryos. We then determined the number of genes in each tissue list that were differentially expressed in MZ*udu*$^{-/-}$ embryos (Figure 2.1 E). We found that of these five tissues, all but the margin were associated with higher the average percentage (6%) differentially expressed genes (Figure 2.1 E).

To determine if differentially expressed genes involved in mesoderm development and patterning tended to be more up or down regulated we constructed heat maps for genes associated with shield and margin (Figure 2.1 F). Genes related to the shield tended to be upregulated (blue) while the margin genes were nearly evenly split between upregulated and downregulated (red) genes. This suggests that Gon4l acts to both activate and inhibit genes during mesoderm development; this result is consistent with our lab's previously published work (Williams et al., 2018)

2.4.2 MZ*udu* embryos fail to pattern the mesoderm
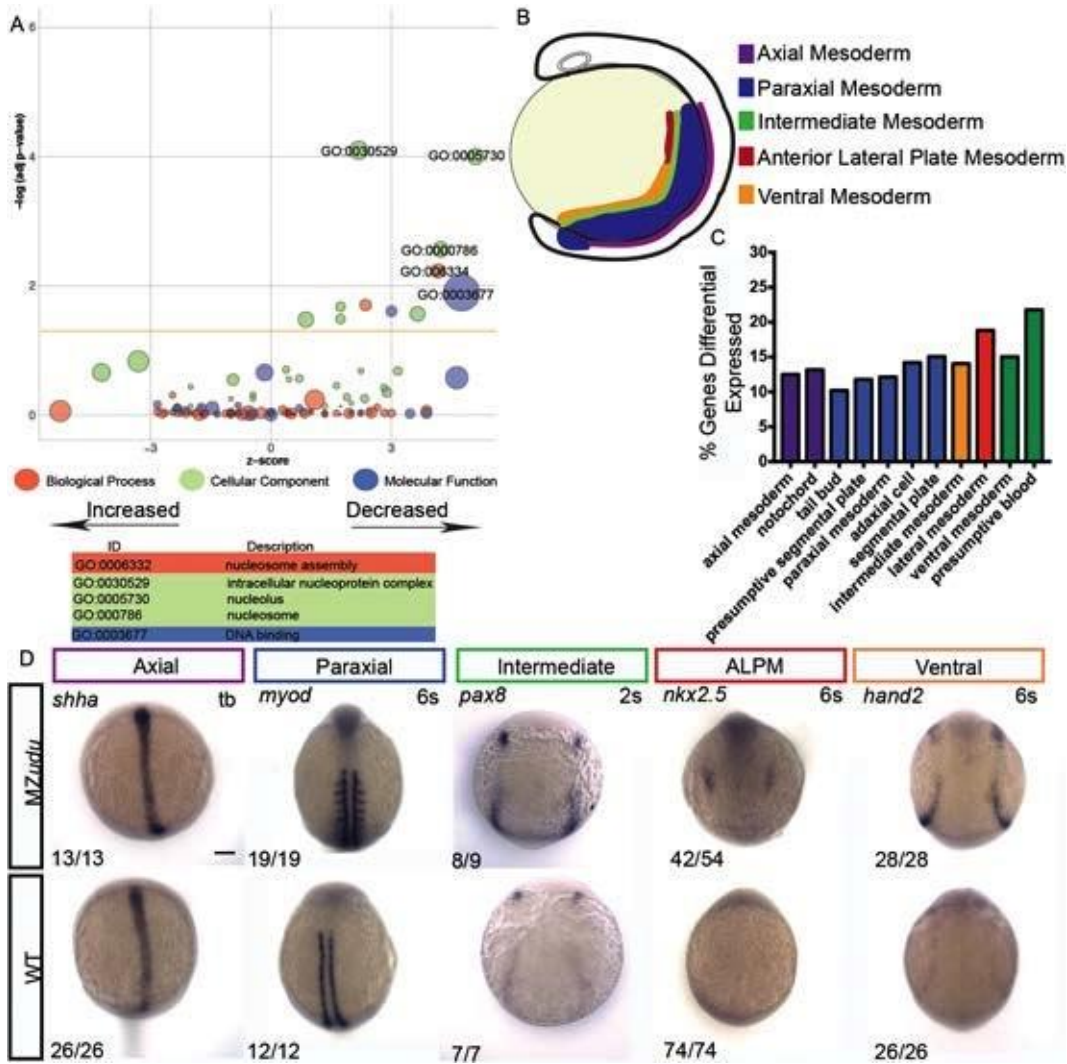
Figure 2.2 Mesoderm patterning is disrupted in MZ*udu* mutant embryos

(A) Plot showing gene ontology enriched terms among the genes differentially expressed in tailbud stage MZ*udu* embryos. Categories shown are biological process (red), cellular component (green), and molecular function (blue). Bubble size represents number of genes per category. Log p-value plotted along Y-axis and Z-score plotted along X-axis. (B) Schematic of the localization of mesoderm sub-types at the start of somitogenesis (C) Graph showing the percentage of genes differentially expressed by mesoderm sub-type: axial (purple), paraxial

(blue), intermediate (green), ALPM (red), ventral (orange). (D) Dorsal view of embryos with WISH for genes for mesoderm patterning at tailbud through early somite stages. Scale bar represent 50μm.

After gastrulation, the mesoderm is organized in the embryo along the DV axis. The axial mesoderm is the dorsal most tissue with the paraxial, lateral plate mesoderm, and intermediate mesoderm radiating from it (Figure 2.2 A) (Schier, 2005). We wanted to test if the early perturbations in mesoderm patterning genes affected the later derivatives of the mesoderm and if particular sub-types were more strongly affected. Our research group previously published an RNA-seq dataset for tailbud stage WT and MZ*udu*$^{-/-}$ zebrafish embryo (Williams et al., 2018). By tailbud stage, the percentage of genes differentially expressed in MZ*udu*$^{-/-}$ embryos has doubled from 5% of genes differentially expressed at shield stage compared to more than 11% of genes at tailbud stage. This suggests that the defects caused by loss of Gon4l become more widespread in later development.

GO analysis examining terms related to molecular function, biological process, and cellular component was performed on the previously published tailbud stage MZ*udu* RNA-seq data sets (Figure 2.2 A) (Huang da et al., 2009a, b; Williams et al., 2018) and identified enrichment in terms related to nucleosomes and DNA-binding. We wondered how the widespread genomic changes affected mesoderm development at tailbud stage through early somitogensis. At these stages of development, the embryo has formed the mesoderm and it is being patterned it into the axial, paraxial, intermediate, anterior lateral plate mesoderm (ALPM), and ventral mesoderm (Figure 2.2 B). Using ZEOGs we annotated genes expressed in mesoderm-derived tissues at tailbud stage and calculated the percentage of these genes that were differentially expressed (Williams et al., 2018). Our analysis found that the majority (n=10/11) of the mesoderm tissues we analyzed had a higher than average (11%) percentage of differentially expressed genes (Figure 2.2 C). In particular, the two regions of the lateral plate mesoderm-anterior and ventral- appeared to be most strongly affected.

Expression domains of genes related to each mesoderm sub-type were evaluated using WISH at stages from tailbud through mid-somitogenesis (Figure 2.2 D). *shh,* a marker for axial mesoderm, appears to be expressed normally in the MZ*udu*$^{-/-}$ embryos (n=26/26) compared to WT (n=13/13) at tailbud stage. Paraxial mesoderm, labeled with *myod*, was also expressed in MZ*udu*$^{-/-}$ embryos (n=12/12), albeit lacking segmental expression consistent with the failure of MZ*udu*$^{-/-}$ embryos to form somites as in WT (n=19/19) at the 6-somite stage. Intriguingly, the expression domains of more ventral mesoderm sub-types: the intermediate, ALPM, and ventral mesoderm were absent or strongly reduced in nearly all of the MZ*udu*$^{-/-}$ embryos. Intermediate mesoderm as marked by *pax8*, was strongly reduced compared to WT embryos (n=8/9) but still present in MZ*udu*$^{-/-}$ embryos (n=7/7) at 2-somite stage. *nkx2.5* expression, which labels the nascent cardiomyocytes in the ALPM, was undetectable in the majority of MZ*udu* mutant embryos (n=72/72) relative to the bilateral domains observed in WT embryos (n=42/54) at 6-somite stage. Ventral and anterior lateral plate mesoderm, marked by *hand2*, are also strongly reduced in the MZ*udu*$^{-/-}$ embryos (n=26/26) compared to WT (n=28/28) at 6-somite stage. These defects in mesoderm patterning are not due to the previously reported roles of Gon4l in regulating *tp53* expression (Liu et al., 2007b), as inhibiting apoptosis through injection of *bcl-xl* is not sufficient to restore either axial or ALPM mesoderm formation (Williams et al., 2018).

These results suggested that while Gon4l is not necessary for mesoderm formation, it is required for later patterning of the mesoderm into its sub-types. Notably, the mesoderm sub-types that would reside more closely to the margin at shield stage are the most intact. We noted that at shield stage the dorsal organizer appears less affected than the ventral organizing signals (Figure 2.1 B).

2.4.3 MZ*udu* embryos present abnormal development of mesoderm derived organs and tissues
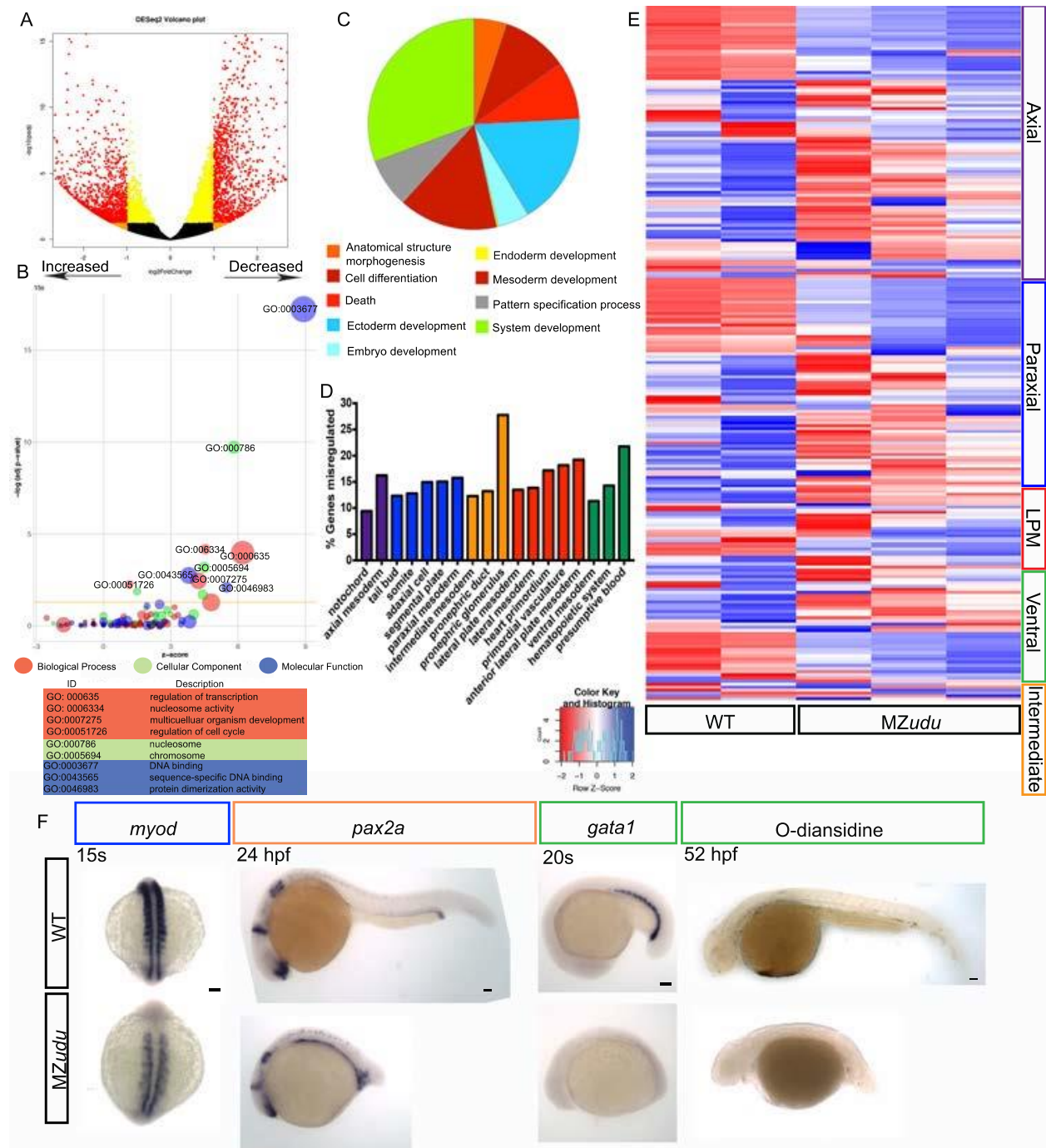
Figure 2.3 MZ*udu* mutant embryos present abnormal development of mesoderm derived organs and tissues

(A) Plot of gene expression changes in MZ*udu* embryos compared to WT at shield stage. Red dots represent genes with significantly different expression levels than WT (p≤0.05 and ≥2 fold change) (B) Plot showing gene ontology enriched terms among the genes differentially expressed in tailbud stage MZ*udu* embryos. Categories shown are biological process (red), cellular component (green), and molecular function (blue). Bubble size represents number of genes per category. Log p-value plotted along Y-axis and Z-score plotted along X-axis. (C) GO enriched terms for differentially expressed genes in the sub-category of developmental process. (D) Graph showing the percentage of genes differentially expressed by mesoderm sub-type: axial (purple), paraxial (blue), intermediate (orange), ALPM (red), and ventral (green). Heat map of genes associated with mesoderm tissues upregulated (blue) and down regulated at the15-somite stage. (F) WISH for organs derived from mesoderm from 15-somite stage through 52 hpf. Scale bars represent 50µm.

Next, we wanted to probe whether early defects in mesoderm patterning in MZ*udu*

mutant embryos affected the later development of mesoderm-derived organs and tissues. We

performed RNA-seq on 15-somite stage MZ*udu*$^{-/-}$ and WT embryos to gain and understanding

how the transcriptome is altered in MZ*udu* mutant embryos at this stage. 2388 genes were

differentially expressed in 15-somite stage MZ*udu* mutant embryos (p≤0.05 and ≥2 fold change),

comprising approximately 9 percent of the zebrafish genome. The differentially genes are split

between up (52%) and down regulation (48%) and further supports our previous work which

showed that Gon4l is capable of both activating and repressing transcription (Williams et al.,

2018) (Figure 2.3 A). Biological process, cellular component, and molecular function analysis

highlights the role of Gon4l in regulating transcription and epigenetics with DNA-binding,

chromosomes, and regulation of transcription among the most highly enriched terms (Figure 2.3

B). Further GO term functional analysis of the differentially expressed genes identified

endoderm, mesoderm, and ectoderms as enriched clusters (Figure 2.3 C). We wanted to focus on

the abnormalities in mesoderm development, so we then analyzed the percent of misregulated

genes per mesoderm-derived tissue and found that the majority (n=15/18) of the categories had a

higher percentage of differentially expressed genes than the average level of 9% (Figure 2.3 D).

ALPM appeared, in general, to have a higher percentage of misregulated genes relative to the

other mesoderm sub-types. We generated a heatmap to visualize the differentially expressed

genes in the different mesoderm sub-types (Figure 2.3 E). Overall, the differentially expressed

genes in the MZ*udu* mutant embryos did not strongly group towards up or down regulated in any

of the mesoderm types. Taken together our RNA-seq and DamID-seq data suggest that Gon4l

both activates and represses genes during mesoderm patterning as we previously showed in

notochord boundary formation (Williams et al., 2018)..

Using WISH and O-diansidine, we probed how the early deviations in mesoderm formation affected the development of mesoderm derivatives at later stages of embryogenesis(Figure 2.3 D) Labeling for *myod*, a marker of somites, was expressed, but lacks somite domains in the MZ*udu* mutant embryos. *pax2a,* a marker for the pronephros is expressed in the expected domains in MZ*udu*$^{-/-}$ embryos and possibly with higher staining intensity than in WT embryos. Markers for blood, *gata1* and O-diansidine, were almost completely absent in MZ*udu* mutant embryos. Together these results suggest that almost all mesoderm derivatives have disrupted gene expression levels. Yet, the phenotypes and severity of the defects in the mesoderm tissues and organs varies among the different types with some appearing normal while others are absent.

2.4.4 Heart development is severely impaired in MZ*udu* mutant embryos
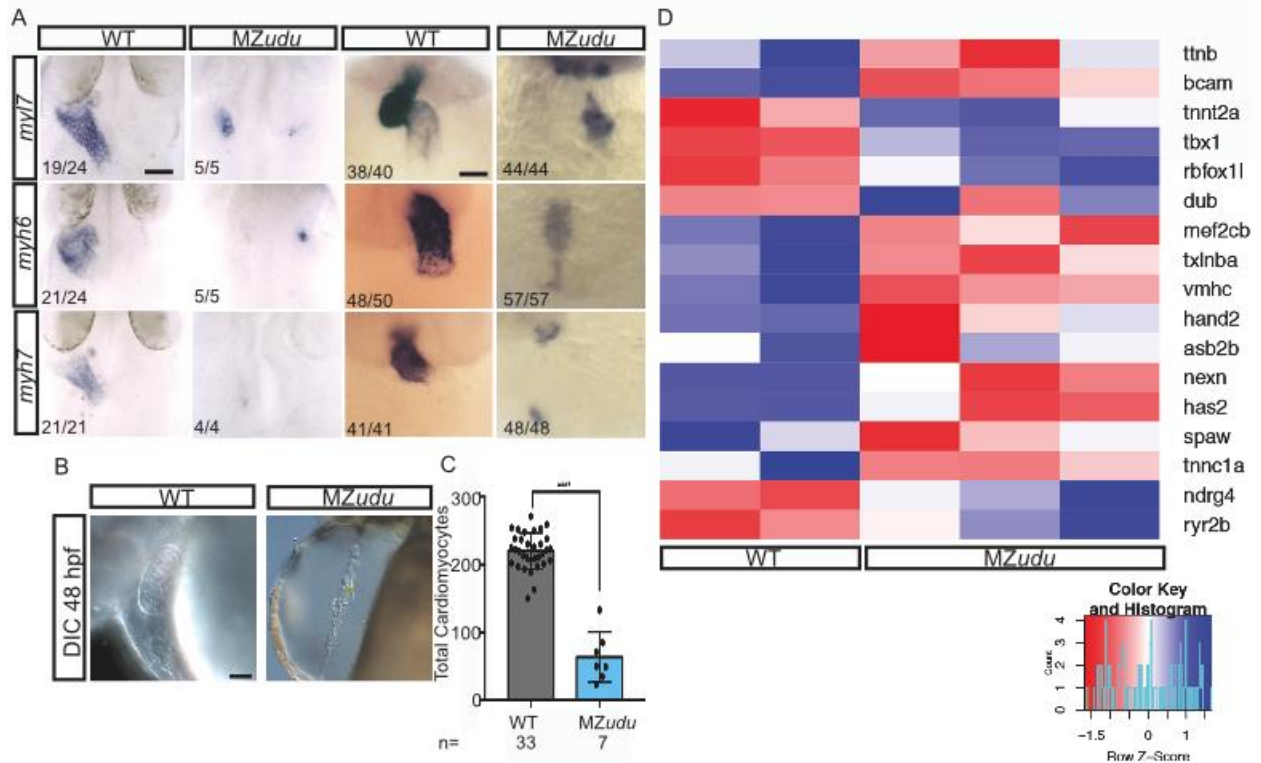
Figure 2.4 MZ*udu* mutant embryos present an almost complete loss of heart development
(A) WISH markers for heart genes at 24 hpf (dorsal view) and 48 hpf (ventral view). (B) DIC lateral view of WT and MZ*udu*$^{-/-}$ embryo hearts at 48 hpf. (C) Graph showing number of cardiomyocytes at 48 hpf in WT (grey) and MZ*udu*$^{-/-}$ embryos (blue). (D) Heat map showing up (blue) and down (red) genes associated with heart primordium from 15-somite stage RNA-seq. (Scale bar represent 50µm.

Our group previously described the heart defects in Z*udu* mutant embryos (Budine et al., *in submission*). Z*udu* mutant hearts form hearts with a smaller number of cardiomyocytes and deficiencies in maintenance of ventricular identity. We, therefore, wondered how MZ loss of Gon4l would influence heart development. First, we examined heart formation using WISH labeling (Figure 2.4 A) and DIC microscopy (Figure 2.4 B). Together, these revealed a severely dysmorphic and smaller heart in the MZ*udu*$^{-/-}$ embryos. Notably, the hearts can beat which suggests that at the MZ*udu*$^{-/-}$ embryos can form at least some mature cardiomyocytes.

At 24 hpf, expression domains of the pan-cardiac *myl7* gene are much smaller in the MZ*udu* (n=5/5) mutant embryos than in WT (n=16/20), and the cardiomyocytes have failed to converge at the midline to assemble the heart tube. Likewise, the atrium specific *myh6* and ventricle specific *myh7* were also expressed in severely reduced domains in the MZ*udu* (n=5/5, n=4/4) mutant embryos at 24 hpf relative the expression domains of WT embryos (n=14/15, n=21/21). At 48 hpf, the expression domains of *myl7, myh6*, and *myl7* were still strongly reduced in MZ*udu* mutant embryos (n=5/5, n=5/5, n=4/4) relative to WT (n=44/44, n=57/57, n=48/48). The MZ*udu*$^{-/-}$ embryos present ectopic development of cardiomyocytes as revealed at both 24 and 48 hpf by *myl7*, *myh6*, and *myh7* WISH (Figure 3 B). The ectopic heart regions observed in mutant embryos likely arise from the failure of the cardiomyocytes to converge from the bilateral heart fields during somitogenesis to the embryonic midline (Figure 2.7) and could be a result of the reduction in endoderm noted at 80% epiboly stage (Figure 2.1 A) (Holtzman et al., 2007; Ye et al., 2015).

To quantify the number of cardiomyocytes, MZ*udu*$^{-/-}$ females were crossed to a *udu*$^{+/-}$ male carrying Tg(*myl7*:eGFP) transgene, which labels cardiomyocytes. Dissected hearts were counter stained with DAPI to enable nuclei counting, and hearts were imaged on a confocal

microscope. Cardiomyocytes were counted by overlaying the GFP channel with DAPI. At 48 hpf

we found that MZ*udu* (63.4±14, n=7) (mean±S.E.M) mutant hearts contained on average fewer

than one third the number of cardiomyocytes as WT (220±4.6, n=33) (p<0.0001).

We were especially interested in the expression of genes involved in heart development

during gastrulation and segmentation. From our 15-somite stage RNA-seq data, we examined the

expression levels of genes associated with the heart primordium and found a widespread

misregulation of these genes (Figure 2.4 D). The differentially expressed genes associated with

heart development tended to be downregulated in MZ*udu*$^{-/-}$ embryos. This finding contrasts with

other mesoderm derivatives, which had more even split between genes with higher and lower

expression levels (Figure 2.4 E).

2.4.5 Gon4l is required both cell-autonomously and non-cell-autonomously in heart
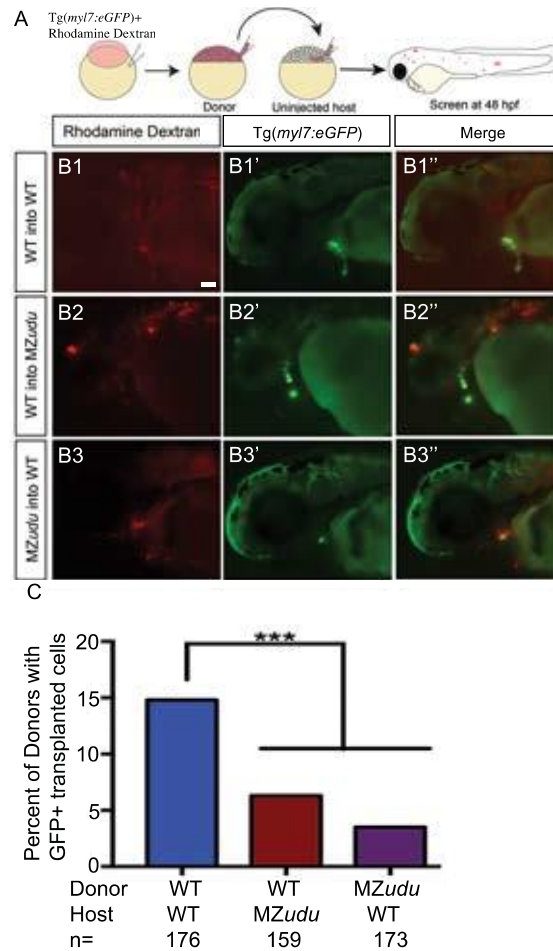
development

Figure 2.5 Gon4l has both cell-autonomous and non-cell-autonomous roles in heart development

Cell autonomy transplants showing incorporation of Tg(*myl7*:GFP) cells into the hearts of

donors. (A) Experiment schematic (B) Images showing the incorporation of rhodamine dextran

(B1-B3), Tg(*myl7:eGFP*) (B1'-B3'), and merged (B1''-B3'') in transplanted host embryos

(lateral view). (C) Graph showing the percentage of hearts from each transplant group that

incorporated transplanted cardiomyocytes into the heart. Scale bar represent 50μm.

Given the abnormal expression of genes encoding both morphogens and cardiac

transcription factors in MZ*udu*^{-/-} embryos in our RNA-seq datasets, we wanted to determine if

the observed cardiac defects arise through cell-autonomous or non-cell autonomous functions of Gon4l. To conduct cell autonomy experiments, MZ*udu*$^{-/-}$ females were crossed to Z*udu* males carrying the Tg(*myl7:eGFP*) (Huang et al., 2003) transgene (Figure 2.5 A). These donor embryos were injected at one-cell stage with rhodamine dextran as a lineage marker. Cells were then transplanted from the margin to the margin of unlabeled host embryos (Figure 2.5 A) (Ho, 1990; Thomas et al., 2008). We first established the baseline rate of transplanted donor cells integration into the donor heart in WT to WT transplants (Figure 2.5 B1-B1''). 14.8% (n=26/176) of hosts had transplanted cardiomyocytes. Next, to test if Gon4l is required non-cell autonomously, we transplanted cells from WT embryos carrying the *myl7*:eGFP transgene into MZ*udu* mutant embryos. We found that at 48 hpf, WT cells in MZ*udu*$^{-/-}$ embryos (Figure 2.5 B2-B2'', C) had a lower rate of incorporation into the heart (n=10/159) than WT cells transplanted into a WT host (Figure 2.5 C) (n=26/176) (p=0.001, chi-square). These results indicated that Gon4l has non-cell autonomous roles in heart development. Then we transplanted MZ*udu*$^{-/-}$ cells into WT hosts. MZ*udu*$^{-/-}$ cells incorporated into the hearts of WT hosts at a lower rate (n=6/173) than WT cells into WT hosts (n=26/176) (Figure 3 B3-B2'') (p=0.0002, chi-square). These results argue for Gon4l having cell autonomous roles in heart development.

In hematopoiesis cell-autonomy transplants in Z*udu* mutant embryos, only a cell-autonomous requirement for Gon4l was discerned (Liu et al., 2007b). Our findings for a non-cell-autonomous role for Gon4l in heart development could reflect a couple of possible explanations. One potential explanation is that Gon4l has different mechanisms in hematopoiesis versus heart development. Perhaps the more likely explanation is that the non-cell-autonomous functions originate in the maternal functions of Gon4l in mesoderm development.

Altogether these results support a model whereby Gon4l regulates heart development through both cell autonomous and non-cell autonomous roles. These findings provide additional support to our studies that find both expression of the early mesoderm patterning genes are disrupted in MZ*udu* mutant embryos as well as the cardiac transcription factors. These transplantation experiments suggest that both the non-cell autonomous patterning defects and cell-autonomous disruptions in cardiac transcription factors contribute to the heart defects in MZ*udu* mutant embryos.

2.4.6 Gon4l Motif Finding

Previously our lab identified potential Gon4l binding sites using DNA adenine methyltransferase identification paired with sequencing (DamID-seq) (Steensel, 2001; Williams et al., 2018). DamID-seq uses a protein of interest fused to an *Escherichia coli* Dam, which methylates adenine residues in the genomic DNA in close physical proximity to the fusion protein (Steensel, 2001). The methylation provides a history of where the protein localized within the genome. Our DamID-seq was performed on RNA collected from tailbud stage embryos (Williams et al., 2018). From these data we were able to predict genes that are potential Gon4l targets.
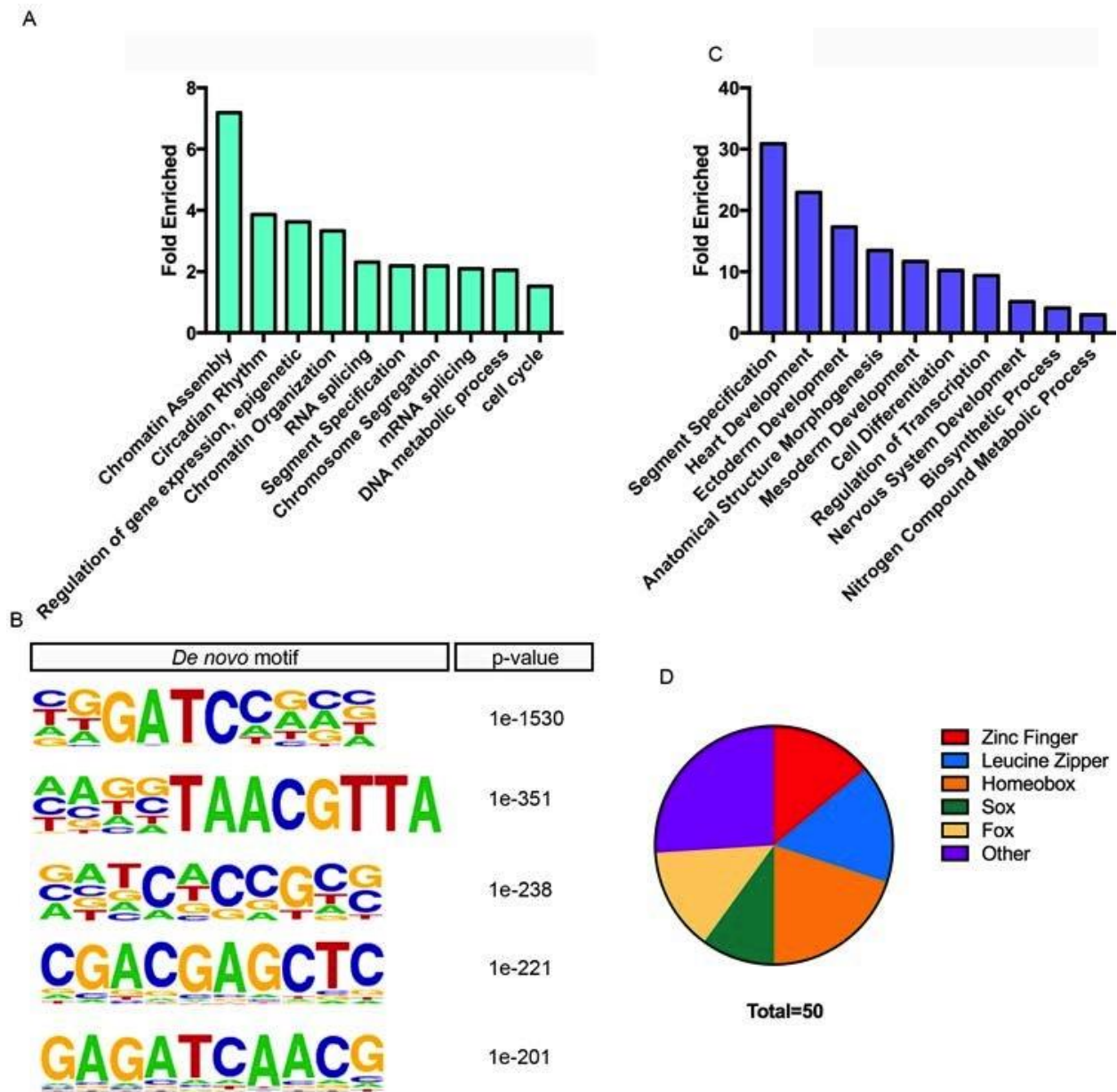
Figure 2.6 DamID-seq analysis and motif finding identify conserved genomic regions of Gon4l association

Figure 2.6. Dam-ID analysis and Motif finding identify conserved genomic regions of Gon4l association. (A) Plot showing fold enrichment for GO terms for Gon4l associated genes in DamID-seq dataset (B) Gon4l association motifs and their p-values. (C) PANTHER GO analysis

for gene function for Gon4l-Dam associated genes (D) Chart showing the distribution of known

the top 50 Gon4l motifs among transcription factor families

We decided to further analyze the DamID-seq data to ask if there are trends related to the types of genes with which Gon4l associates in the genome. We performed functional annotation for biological processes using PANTHER and found enrichment in terms related to chromatin assembly and epigenetic regulation of genes (Figure 6 A)(Thomas et al., 2006). This is consistent with our RNA-seq datasets showing that differentially expressed genes are enriched for DNA-binding and transcriptional clusters (Figure 2.6 A). It is also consistent with the RNA-seq datasets from shield, tailbud, and 15-somite stage embryos that found enrichment of differentially expressed genes related to DNA regulation and chromosomes (Figures 2.1, 2.2, 2.3).

Next, we wondered if there were conserved motifs for Gon4l binding. We used HOMER to identify *de novo* enriched DNA sequence motifs from the DamID-seq dataset (Figure 2.6 B) (Heinz et al., 2010). The *de novo* motif sequences were compared to known DNA sequence motifs of transcription factors and the closest matches to the *de novo* Gon4l motifs identified. We also performed functional annotation of the transcription factors to known motifs and identified the closest matches; these transcription factors were highly enriched for functions in segment specification, hearts development, and mesoderm (Figure 2.6 C). The motif for Yy1, a known binding partner of Gon4l (Lu et al., 2010), was among the known motifs that most closely matched the Gon4l *de novo* motifs, which lends further credence to the motifs identified ($p<<<0.0001$). This result suggests that Gon4l preferentially localizes in the regions of the genome near binding sites for transcription factors that regulate patterning of the mesoderm and its derivatives.

Then we probed if there was enrichment for the Gon4l motifs among different transcription factor structure classes (Figure 2.6 D). For the top 50 known motifs, we determined

the structural class of the transcription factor. 20% of the top 50 motifs were associated with Homeobox (HOX) transcription factors. Homeobox transcription factors have roles in embryonic development, particularly in segment specification and individual expression patterns in organogenesis (Pick, 2016). Other highly represented classes of transcription factors among the motifs were leucine zipper, zinc finger, SOX and FOX. SOX and FOX class transcription factors have well characterized roles in embryonic development and function in the differentiation of all three germ layers (Golson and Kaestner, 2016; Sarkar and Hochedlinger, 2013). Altogether, our motif analysis argues that Gon4l associates with genomic regions enriched for developmentally important transcription factor association sites. This association encompasses broad functional and structural classes of transcription factors. Together, these results suggest that Gon4l could have broad roles in modulating the expression of developmentally important genes during development and could be cooperating with many types of transcription factors to do so.

2.5 Discussion

Here we show the requirement for Gon4l in germ layer formation and throughout mesoderm development through embryologic, gene expression and genomic analyses. In MZ*udu* mutant embryos, germ layer formation occurs relatively normally. However, subtle defects in mesoderm and endoderm can be discerned in MZudu$^{-/-}$ embryos during early gastrulation stages. Loss of maternal and zygotic Gon4l function results in defects in DV patterning of mesoderm in zebrafish embryos and ultimately results in an abnormal development of most mesodermal tissues in the embryos. These defects are more severe than those previously noted in zebrafish embryos lacking only zygotic Gon4l (Lim et al., 2009; Liu et al., 2007b). The

results reported here indicate that Gon4l has essential roles in germ layer induction, mesoderm patterning, and the development of mesoderm-derived organs.

Through RNA-sequencing we demonstrate MZ loss of Gon4l causes broad misregulation of the genome, particularly in genes related to DNA-binding, chromosome, and mesoderm during zebrafish embryogenesis. The differentially expressed genes are almost equally split between upregulated and downregulated genes. These expand our early finding that Gon4l can both activate and repress gene expression (Williams et al., 2018).

The phenotypes reported here implicate Gon4l in the development of an array of mesoderm derivatives. Intriguingly, lateral plate mesoderm with its constitutive anterior and ventral components, which gives rise to the heart and blood respectively, appears to be among the most strongly disrupted mesoderm sub-types. Heart development in MZ*udu* embryos is strongly disrupted with hearts containing less than one quarter the number of cardiomyocytes as WT embryos, and the cardiomyocytes fail to converge into a beating heart tube by 24 hpf. RNA-seq found that differentially expressed genes related to the heart primordium tend to be downregulated. This suggests that Gon4l could have a role in inducing expression of heart-related genes.

Through cell-autonomy transplants we demonstrate the Gon4l has both cell-autonomous and non-cell-autonomous roles in heart development. The non-cell-autonomous roles could be in defining the cardiac competent mesoderm during early gastrulation. Previously, Gon4l had been shown to act cell-autonomously during zygotic blood development (Liu et al., 2007b). This could mean that the non-cell-autonomous roles arise through the maternal functions of the protein. The cardiac competent mesoderm is defined through a variety of secreted signaling pathways including *wnt*, *bmp*, and retinoic acid that work together to endue a subset of mesoderm cells

with the competency to become cardiomyocytes (Bakkers, 2011; Liu and Stainier, 2012; Ueno, 2007). The cell-autonomous roles could result from both Gon4l regulating proliferation and modulating the expression of cardiac transcription factors (Lim et al., 2009; Liu et al., 2007b; Lu et al., 2010). In our group's previously published Gon4l DamID-seq data set, we find that Gon4l associates with the regulatory regions of cardiac transcription factors and cell-cycle regulatory genes, among many other genes.

Motif analysis of the DamID-seq data identified *do novo* motifs for Gon4l association within the genome. When these Gon4l motifs were compared to known motifs of transcription factors, we discovered an enrichment in motifs for transcription factors related to segment specification and heart development. This finding could indicate that Gon4l forms complexes with transcription factors involved in these processes. Gon4l has been shown to bind Yy1, Sin3a, and Hdac1 in mouse (Lu et al., 2011), but additional binding partners have not yet been identified. Gon4l forming complexes with different transcription factors could help explain the vast roles of the protein.

Together with our previous work, this study defines the roles of Gon4l in germ layer induction and mesoderm patterning. This study provides analysis of the categories of genes differentially expressed in MZ*udu* mutant zebrafish embryos and demonstrates that loss of Gon4l results in severe disruption of mesoderm tissues patterning. It is important to note that endoderm and ectoderm development also appear to be impacted in MZ*udu* mutant embryos, but these are not analyzed in depth here. It is conceivable that defects in endoderm development contribute to the mesoderm defects describe herein, particularly in the merging of the bilateral cardiac fields. Future experiments will need to be undertaken to elucidate the genetic and phenotypic deficiencies that arise in the development of ectoderm and endoderm.

We demonstrated that both cell-autonomous and non-cell-autonomous roles of Gon4l are required for the proper development of mesoderm-derived tissues. Finally, novel motif analysis provides evidence that Gon4l preferentially associates with genomic regions where transcription factors involved in segment specification and heart development bind. In conclusion, we demonstrate that maternal and zygotic loss of Gon4l results in loss of mesoderm derived tissues and that the protein is essential for regulating genes that pattern the mesoderm.

2.6 Supplemental Information
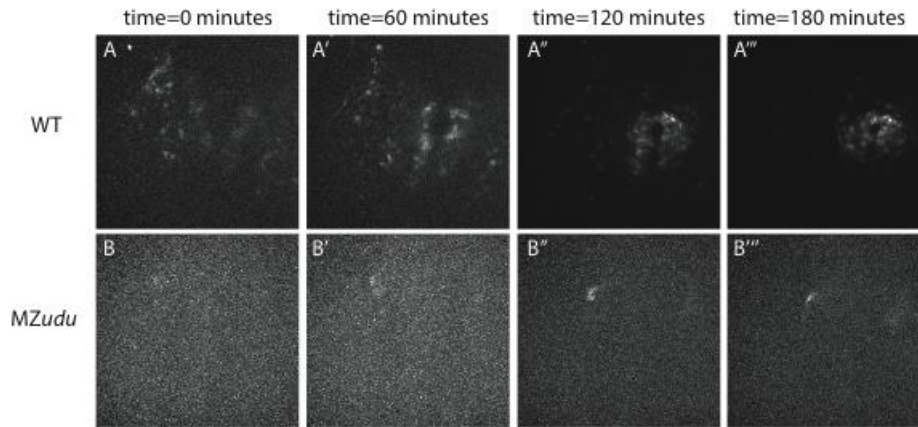
Figure 2.7 Cardiomyocytes fail to converge at the midline in MZudu mutant embryos

Cardiomyocytes in WT embryos (A-A''') and MZ*udu* embryos (B-B''') from 16-somite stage

through16-somite stage+180 minutes.

Chapter 3: Gon4l/Udu Regulates Cardiomyocyte Proliferation and Maintenance of Ventricular Chamber Identity During Zebrafish Development

3.1 Abstract

Vertebrate heart development requires temporal regulation of gene expression to specify cardiomyocytes, increase the cardiomyocyte population through proliferation, and establish and maintain the atrial and ventricular cardiac chambers identity. The evolutionarily conserved chromatin factor Gon4l encoded by the zebrafish *ugly duckling* (*udu*) locus has previously been implicated in cell proliferation as well as specification of mesoderm-derived tissues including blood and somites, but its role in heart formation has not been studied. Here we report two distinct roles of Gon4l/Udu in heart development: cell proliferation and maintenance of ventricular chamber identity. We show that zygotic loss of *udu* function results in a small decrease in cardiomyocyte number at 1 day post fertilization that becomes exacerbated during later development. We present evidence that the cardiomyocyte deficit in *udu* mutants is due to reduced proliferation and not the TP53-dependent apoptosis implicated in hematopoietic deficiencies. Accordingly, expression of the G1/S-phase cell cycle regulator *cyclin E2* is reduced in *udu* mutant hearts and DNA adenine methyltransferase identification with sequencing demonstrated Gon4/Udu to be associated with *cyclin E2* genomic sequences. Furthermore, *udu* mutant hearts manifest a reduction of ventricular fate, a phenotype associated with progressive reduction of *nkx2.5* expression. Analyses of *udu;nkx2.5* compound mutant phenotypes reveal that *udu* and *nkx2.5* genes interact in maintenance of ventricular identity. Finally, we show that

ectopic expression of *nkx2.5* is not sufficient to restore ventricular chamber identity suggesting that Gon4l acts on multiple genes within the cardiac chamber-patterning pathway. Together, our findings define a novel role for Gon4l in coordinating cardiomyocyte proliferation and chamber identity during heart development.

3.2 Introduction

The heart is the first organ to form and function in the developing embryo (Bakkers, 2011). Defects in early heart development can result in congenital heart disease (CHD), which is the most common birth defect affecting nearly 1% of newborns (Pierpont et al., 2007). While much progress has been made in identifying the complex gene networks that specify, pattern and shape the heart, the role of chromatin factors and how they regulate these networks are less well understood. Some of the epigenetic modifiers of heart development have been described such as Brg1 of the BAF complex and the histone methyltransferase Jmjd3, but there likely remain many unknown epigenetic regulators (Lickert, 2004; Ohtani et al., 2013; Takeuchi et al., 2011). Advancing knowledge of the gene regulatory cascade underlying normal heart development is essential for better diagnosis, treatment and prevention of CHDs.

The heart develops rapidly in zebrafish embryos, which can survive up to five days without a functioning cardiovascular system, making it an ideal model system in which to study cardiogenesis (Bakkers, 2011). By 24 hours post fertilization (hpf), a beating heart tube is formed. Over the next 24 hours, the heart tube undergoes a series of gene expression and morphology changes that form and pattern the atrium and ventricle (Yelon, 1999). These two cardiac chambers expand in size through *de novo* differentiation of cardiac precursor cells (CPCs) and proliferation of existing cardiomyocytes (Foglia and Poss, 2016). Several cardiac-

specific transcription factors involved in cardiomyocyte specification and patterning of the cardiac chambers have been identified, including Hey2, Irx4, Tbx5 and Nkx2.5 (Bao, 1999; Bruneau, 1999; Bruneau et al., 2001; George et al., 2015; Stainier, 2001; Yutzey, 1994, 1995). Interestingly, loss-of-function mutations in these cardiac genes often lead to reduced expression of ventricle-specific genes, suggesting that atrial fate might be a default state (Stainier, 2001; Yutzey, 1994, 1995). Despite the advances made in understanding the genetic cascades underpinning heart development, it is still unclear how these genes are regulated and their expression coordinated.

Nkx2.5 is of particular interest among these transcriptional regulators as it plays critical roles throughout cardiac development (Harvey, 2002; Targoff et al., 2013). This evolutionarily conserved transcription factor is required for cardiogenesis in fruit flies (Bodmer, 1993), and its homologues in other species including mouse, zebrafish, and human have essential functions in heart development (Balci and Akdemir, 2011; Benson et al., 1999; Bloomekatz et al., 2017; Elliott et al., 2003; Grow, 1998; Jay, 2003; Lyons, 1995; McElhinney et al., 2003; Schott, 1998; Tanaka, 1999; Targoff et al., 2008; Ueyama et al., 2003). In zebrafish, *nkx2.5* and its paralog *nkx2.7* are expressed in the nascent cardiomyocytes during early development, (Chen and Fishman, 1996; Reifers et al., 2000) and are later required for maintenance of ventricular identity (Targoff et al., 2013; Targoff et al., 2008). Loss of *nkx2.5* expression results in a heart with reduced expression domains of ventricular genes, and simultaneous inactivation of *nkx2.7* further enhances the deficiency of ventricular identity and leads to atrial cell-fate expansion (Targoff et al., 2013; Tu et al., 2009). Overexpression of *nkx2.5* can compensate for loss of both *nkx2.5* and *nkx2.7* indicating that the genes have largely overlapping functions (George et al., 2015; Tu et al., 2009)

How cell fate specification, patterning, morphogenesis, and proliferation are coordinated throughout cardiac development remains an outstanding question in the field. Recent studies point to transcriptional regulation of cardiac transcription factors and proliferation by epigenetic modification, providing a potential mechanism for coordinating cardiac development (Gregoire et al., 2013; Jung et al., 2005; Nakajima et al., 2011).

The zebrafish *ugly duckling (udu)* locus, which encodes the chromatin factor Gon4l, was first identified for its role in tail morphogenesis (Hammerschimidt et al., 1996; Liu et al., 2007b). Subsequent work in zebrafish defined roles for Gon4l in somitogenesis and (Lim et al., 2009; Liu et al., 2007b). These defects in zebrafish *udu* mutant embryos were partially ascribed to abnormal cell cycle progression and increased TP53-mediated apoptosis (Lim et al., 2009; Liu et al., 2007b). Hypomorphic *Gon4l* mutations in mouse result in a failure of B-cell development and cell cycle arrest, demonstrating a conserved role in blood development (Barr et al., 2017; Lu et al., 2010). Structural analysis of the murine Gon4l protein, identified putative SANT and Yy1-binding domains (Liu et al., 2007b; Lu et al., 2010). SANT domains are found in chromatin remodeling complexes (Boyer, 2004), and Yy1-binding domains enable interactions with the transcription factor Yy1, which acts as both a negative and positive regulator of gene expression (Deng, 2010; Gregoire et al., 2013). Studies in mouse have also elucidated the ability of Gon4l to bind HDAC1 and Yy1 (Lu et al., 2011), both of which are known epigenetic regulators of *nkx2.5* and other cardiac genes (Lu et al., 2011; Nan and Huang, 2009).

Despite Gon4l's importance in the development of mesoderm-derived tissues, including somites and blood (Lim et al., 2009; Liu et al., 2007b; Williams et al., 2018) the role of Gon4l in heart development has not been addressed. Here we show that Gon4l plays essential roles in patterning the cardiac chambers and regulating cardiomyocyte cell cycle during zebrafish heart

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development. Zygotic *udu* mutant embryos present a slight reduction in the total number of

cardiomyocytes at 24 hpf. However, the number of cardiomyocytes in *udu* mutant did not

increase after 24 hpf, which was due to reduced proliferation, caused by cell cycle disruption.

Moreover, at 48 hpf *udu* mutant hearts exhibited expansion of atrial at the expense of ventricular

fates. Our studies demonstrate that *udu* genetically interacts with *nkx2.5* to maintain ventricular

identity. This work describes Gon4l as a novel modulator of heart development that regulates

both cardiac chamber fate maintenance and proliferative decisions.

3.3 Materials and Methods

Zebrafish

Zebrafish were housed and handled according to protocols approved by the Institutional Animal

Care and Use Committee of Washington University School of Medicine. AB* or AB*/Tubingen,

Tg(*hsp70l:nkx2.5-eGFP*) (George et al., 2015), *Tg(myl7:GFP)* (Huang et al., 2003), *udu*[vu66]

(Williams et al., 2018), *nkx2.5*[vu179] (Targoff et al., 2013), and *tp53*[zdf1] (Berghmans et al., 2005)

were used. Fish were fed with rotifers during larval stages and rotifers and dry food during

adulthood. Embryos were produced through natural matings, maintained at 28.5°C, and staged

according to Kimmel's embryonic stages (Kimmel, 1995).

Whole-mount in situ hybridization (WISH)

Embryos were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline

(PBS). Embryos older than 24 hpf were bleached after fixation until pigment was removed in a

solution containing 3% H_2O_2 and 0.5% KOH prior to carrying out the WISH (Thisse and Thisse,

2008). WISH was performed as previously described using 65% formamide solution and 70°C hybridization temperature (Thisse and Thisse, 2008). *Cardiac myosin light chain 7 (myl7)* (Yelon et al., 1999)*, atrial myosin heavy chain (myh6)* (Yelon et al., 1999) and *ventricular myosin heavy chain (myh7) (*Yelon, 2000*)* were used at 500 ng/µL, *nkx2.5* at 650 ng/µL (Chen and Fishman, 1996), *nkx2.7* at 150 ng/µL (Reifers et al., 2000). Stained embryos were cleared in 80% glycerol (Pradhan et al., 2017).

O-diansidine Staining

Embryos were euthanized with 3-amino benzoic acidethylester (tricaine),then placed in a mixture containing O-diansidine (0.06g/mL), sodium acetate (0.01M, pH 4.5), hydrogen peroxide (0.65%) and 40% ethanol, and incubated in the dark at room temperature for 15 minutes (mins) (Detreich W., 1995). Embryos were cleared in 80% glycerol before imaging.

Heart Dissection and DAPI Labeling

Embryos were euthanized in tricaine, and hearts were manually extracted using 27G needles as previously described (Yang and Xu, 2012). Dissected hearts were fixed in 4% PFA for 20 mins and then washed 3 times in PBS. Hearts were labeled with 2-(4-Amidinophenyl)-1H-indole-6-corboxamidine (DAPI) for 3 mins and then washed twice with PBS before being mounted in 2% methylcellulose for imaging.

Immunohistochemistry

For anti-Caspase-3 (Casp3, Cell Signaling, catalogue number:9661S) antibody labeling, hearts were manually dissected as described above and immunostained as previously described (Burns

et al., 2005; Yang and Xu, 2012). Briefly, dissected hearts were fixed in 4% PFA, washed in PBT, and then blocked in 10% goat serum for 1 hr at room temperature. The primary antibody was added (1:200) for 1 hr, whereas the secondary antibody (Life Technologies, catalogue number: A11036) (1:400) for 30 mins, both at room temperature.

MF20 (Developmental Studies Hybridoma Bank, AB_2147781), S46 (Developmental Studies Hybridoma Bank, AB_528376) and Mef2 (Abcam, ab64644) labeling was performed in zebrafish embryos at 24 and 48 hpf (Alexander, 1998; Targoff et al., 2013). Embryos were euthanized in tricaine and then fixed for 1 hr in 1% PFA in PBS, permeabilized for one hour in 0.5% Triton-X100 and 10% goat serum in PBS. Embryos were incubated overnight in primary antibodies (1:20 for S40 and MF20, 1:200 for Mef2), then washed 3 times with PBS and incubated in secondary antibodies (Invitrogen, catalogue numbers: A21121, A21144, A21245) (1:200) for 2 hrs at room temperature.

EdU Labeling

Embryos were placed in a 12.5% 5-ethynyl-2'-deoxyuridine (EdU), 7.5% dimethyl sulfoxide (DMSO) solution in 0.3X Danieau and incubated for 1 hr on ice. Embryos were washed with 0.3X Danieau for 12 hrs at 28.5°C (Just S., 2016), and then euthanized with tricaine. Hearts were manually dissected and fixed for 20 mins in 4% PFA (Yang and Xu, 2012). The dissected hearts were briefly rinsed 3 times with 3% bovine serum albumin (BSA) in PBS and then permeabilized for 30 mins in 0.25% Triton-X100 and 1% DMSO in PBS. The hearts were then incubated for 30 mins in the Click-It Reaction Buffer, washed 3 times in 3% BSA in phosphate buffer saline (PBT) and labeled with Mef2 antibody as described above.

TUNEL

TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling)

was performed as previously described (Williams et al., 2018). Briefly, 48 hpf zebrafish

embryos were fixed overnight in 4% PFA. Embryos were dehydrated using a series of

methanol/PBT washes and stored at -20°C at least overnight. Embryos were then rehydrated

using a series of methanol/PBT solutions, permeabilized with proteinase K and ApopTag

TUNEL (Millipore Sigma, Catalogue number: S7100) assay was performed according to the

instructions provided.

Heat shock

Embryos obtained from natural crosses of $udu^{+/-}$;Tg($hsp70l$:$nkx2.5$-$eGFP$)$^{+/-}$ to $udu^{+/-}$ (George et

al., 2015) were maintained at 28.5°C. At 21-somite stage, 50 embryos were transferred to 1.5 mL

of embryo medium into a heat block held at 37°C for one hour. Transgenic embryos were

identified by ubiquitous GFP expression. Non-transgenic siblings exposed to heat shock served

as experimental controls.

Quantitative Real-Time (qRT) PCR

Total RNA was extracted, 400-600 36 hpf Tg($myl7$:GFP) transgenic embryos were euthanized

using tricaine. Hearts were dissociated in L-15 media using gel loading pipette tips, and GFP

fluorescence was used to manually isolate the population (Lombardo et al., 2015). Hearts were

briefly washed with L-15 media supplemented with 10% BSA and total RNA was extracted

using Trizol (Ambion) with phenol/chloroform extraction.

cDNA was synthesized using the iScript Kit (BIO-RAD, catalogue number: 1708841) using

1,000 ng of RNA per 20 μL reaction. qRT-PCR was performed using SYBR green (Bio-Rad)

using CFX Connect Real-Time machine with a minimum of three independent biological and

technical samples. The following primers were used:

ccnd1 F: 5'-TGGGATCTGGCCTCAGTGAC-3'

ccnd1 R: 5'-TGAAGTTGACGTCTGTCGCAC-3'

ccnd2a F: 5'-AGCCGTATTAAAGGTCGAAAAGG-3'

ccnd2a R: 5'-CCTCGCAGACCTCTAACATCCA-3'

ccnd2b F: 5'-ACTGCTGTGGGAGTTGGTGG-3'

ccnd2b R: 5'-AAGGTTTGCGTGTGCTTGCG-3'

ccnd3 F: 5'-CATCGCCCTCACGGCTACAG-3'

ccnd3 R: 5'-ACATGCAGAGAACGCCTTGTCC-3'

ccne1 F: 5'-TCAGGGCTGAAGTGGTGTGA-3'

ccne1 R: 5'-GGAGTGAACCTTTCCCAGCC-3'

ccne2 F: 5'-GCACTGGACACTGCGGACAA-3'

ccne2 R: 5'-GGGACTCTTCTATTGCACTCGCC-3'

nkx2.5 F: 5'-CCGGATCCTCTCTTCAGCG-3'

nkx2.5 R: 5'-CCTGACAAAACCCGATGTCTTT-3'

nkx2.7 F: 5'-GCTTCAGTGTATGCAGAACACCC-3'

nkx2.7 R: 5'-CGGGGCCGAAAGGTATCTCTGC-3'

Genotyping

DNA was extracted from whole embryos in a solution containing 10 mM Tris-HCL (pH 8), 50

mM KCL, 0.3% Tween-20, 0.3% NPA and 10 mg/mL proteinase K. Embryos subjected to

WISH were treated with NaCl to reverse DNA crosslinking prior to DNA isolation (Gansner et

al., 2008). The following primers were used:

udu^{vu66} F: 5'-GCACTTGCACAAACAGAGTTCCCGTA-3'

udu^{vu66} R: 5'-CAAATTAACTACACGGGACAGCAAC-3'

Followed by MaeIII digestion

p53^{zdf1} mutant F: 5'-AGCTGCATGGGGGGGGAA-3'

p53 WT F: 5'-AGCTGCATGGGGGAT-3'

p53 R: 5'-GATAGCCTAGTGCGAGCACACTCTT-3'

nkx2.5^{vu172} F: 5'-TTACCATCCCGAACCAAAAC-3'

nkx2.5^{vu172} R: 5'-CAAACTCACCTCCACACAGG- 3'

Followed by HinfI digest

Tg(*hsp70l:nkx2.5-eGFP*) F: 5'-*TCACCTCCACACAGGTGAAGATCTG*-3'
Tg(*hsp70l:nkx2.5-eGFP*) R: 5'-*GGGTCAGCTTGCCGTAGGTGG*-3'

Image Analysis

FIJI was used to visualize and manipulate all microscopy images. For dissected hearts labeled

with Tg(*myl7*:*GFP*), DAPI, EdU, Caspase, Mef2, MF20, S46 and anti-pH3 antibody, multiple Z-

planes were imaged and projected to visualize the heart. Cardiomyocytes transgenic images and

immunostainings were manually counted. Figures were prepared in Photoshop CS 6.

Statistical Analysis

Statistical tests were conducted with GraphPad Prism v. 6. To compare the means of number of

cardiomyocytes, proliferative index, apoptotic index and fold difference we used two-tailed,

unpaired Student's t-test and ANOVA. $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Zygotic mutations in *udu* result in reduced cardiomyocyte number and decreased ventricular fate

Although the nonsense *udu^{vu66}* allele in the zebrafish homolog of *ugly duckling/gon4l* locus was

identified as a mutation during a forward genetic screen as a recessive enhancer of shortened

body axes in noncanonical Wnt/Planar Cell Polarity (Wnt/PCP) mutant *gpc4/knypek (kny)*

embryos (Williams et al., 2018), we observed that zygotic *udu^{vu66/vu66}* embryos (*udu^{-/-}* mutants)

had small, dysmorphic hearts with severe edema at 48 hpf (Figure 3.8 A-D). Given the

established roles of Gon4l in hematopoiesis and mesoderm patterning (Barr et al., 2017; Lim et

al., 2009; Liu et al., 2007b; Lu et al., 2011; Lu et al., 2010; Williams et al., 2018), we wanted to

interrogate the functions of Gon4l in heart development.

To characterize cardiac development in *udu* mutant embryos, we first performed whole

mount *in situ* hybridization (WISH) for the cardiac markers *myosin light chain 7* (*myl7)* (Yelon

et al., 1999), *myosin heavy chain 6 (myh6)* (Yelon et al., 1999) and *myosin heavy chain* 7 (*myh7)*

(Yelon, 2000) in 24 hpf embryos to examine overall heart tube morphology and initial patterning

of the atrium and ventricle (Figure 3.1 A-C, E-G) (Yelon, 2000). The heart tube did not appear to

fully extend in the majority of *udu*$^{-/-}$ (n=19/21) embryos (Figure 3.1 A, E). WISH also revealed

that *myh6* (n=15/16) and *myh7* (n=11/12) were expressed in slightly abnormal domains

(Figure3.1 B, F, C, G) compared to WT (de Pater et al., 2009).

| WT 24 hpf | $udu^{-/-}$ 24 hpf | WT 48 hpf | $udu^{-/-}$ 48 hpf |

A *myl7* 19/24
E 19/21
I 38/40
M 37/38

B *myh6* 21/24
F 15/16
J 48/50
N 30/32

C *myh7* 21/21
G 11/12
K 41/41
O 40/41

D MF20/S46/Mef2 n=13
H n=16
L n=13
P n=16

Q
R
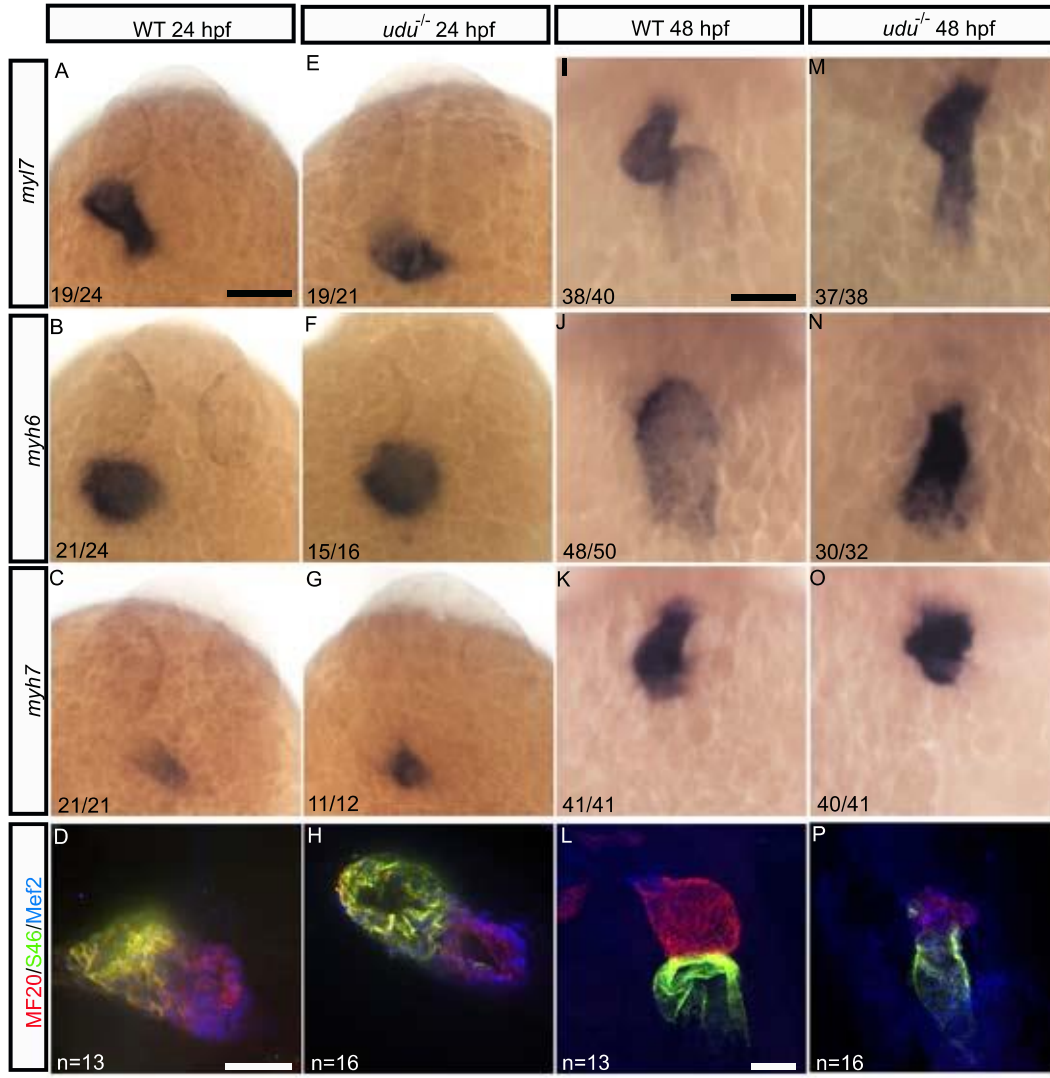
Figure 3.1 Loss of zygotic *udu* affects cardiac chamber patterning and results in a reduction in the number of cardiomyocytes

Dorsal view WT (A-C) and *udu*^{-/-} embryos (E-G) showing the expression of *myl7, myh6,* and *myh7* at 24 hpf by WISH. Immunofluorescence of cardiac chambers at 24 hpf using MF20 (green, atrium) and S46 (red, ventricle) and Mef2 (blue, cardiomyocyte nuclei) antibodies to mark cardiac chambers in WT (D) and *udu*^{-/-} (H) embryos. Ventral view of WT (I-K) and *udu* mutant (M-O) embryos, in which expression of *myl7, myh6,* and *myh7* was by visualized WISH. Immunofluorescence of cardiac chambers at 48 hpf using MF20, S46, and Mef2 antibodies in WT (L) and *udu*^{-/-} (P) embryos. Quantification of cardiomyocytes in WT (grey) and *udu*^{-/-} embryos (blue) at 24 hpf and 48 hpf (Q). Percentage of total cardiomyocytes with ventricular identity 24 hpf and 48 hpf in WT (grey) and *udu*^{-/-} (blue) embryos (R). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars=SEM. Arrow marks atrial fate expansion. Scale bars represent 50μm.

We next examined heart size and cardiac chamber patterning using MF20 and S46 antibody labeling to visualize morphology of the cardiac chambers together with Mef2 (blue), which labels cardiomyocyte nuclei (Yelon et al., 1999). Ventricular myocardium is indicated by MF20 (red) immunofluorescence signal in the absence of S46 (green). At 24 hpf, we found a decrease in the total of cardiomyocytes in *udu* mutant embryos (105±4.6, n=16) relative to WT (130±3.1, n=13) (Figure 3.1 D, H, Q). However, we found that the percentage of cardiomyocytes with antibody labeling consistent with ventricular identity was unchanged between WT (53±2.0, n=11) and *udu* (47±3.0, n=12) mutant embryos (p=0.14) (Figure 1 R).

Next, we asked how loss of Gon4l function affects heart development after heart tube formation. To this end we performed WISH for *myl7, myh6* and *myh7* in *udu*$^{-/-}$ embryos at 48 hpf and found that overall heart size was reduced with both the atrium and ventricle being affected. We observed that at this stage, expression of *myl7, myh6* and *myh7* were strongly reduced in almost all *udu*$^{-/-}$ mutants (Figure 1 M-O) (n=37/38, n=30/32, n=40/41) compared to WT embryos (Figure 1 I-K) (n=38/40, n=48/50, n=41/41). To quantity cardiomyocytes we used Mef2 antibody labeling. The reduction in cardiomyocyte number was greatly exacerbated at 48 hpf, with WT embryos containing 204±4.9 (n=13) and *udu*$^{-/-}$ which contained only 108±5.8 (n=16) (p=0.0001) (Figure 3.1 L, P, Q). Notably, there was an insignificant increase in the number of cardiomyocytes in *udu* mutant embryos between 24 hpf (105±4.6) and 48 hpf (108±5.8) (p=0.70), suggesting that the cardiomyocyte population does not expand after 24 hpf in *udu* mutant embryos. In contrast to the static cardiomyocyte population in *udu*$^{-/-}$ embryos, the cardiomyocyte population in WT embryos increased by more than 50 percent between 24 and 48 hpf (Figure 3.1 Q).

To interrogate whether cardiac chamber patterning is perturbed at 48 hpf we employed MF20, S46, and Mef2 antibody labeling (Yelon et al., 1999) in the 48 hpf embryos. Ventricular myocardium was reduced in $udu^{-/-}$ embryos (Figure 3.1 P, R) compared to WT (Figure 3.1 L, R). In contrast, S46 antibody, which marks atrial myocardium, labeled an expanded domain in $udu^{-/-}$ hearts (Figure 1 P). We quantified this difference in chamber patterning by counting Mef2 labeled nuclei and found that the percentage of total cardiomyocytes that are ventricular in reduced in $udu^{-/-}$ hearts (41±2.0, n=16) relative to WT (50±1.0, n=13) (p=0.0009) (Figure 3.1 Q).

These observations indicate that loss of Gon4l function results in cardiac defects impacting both the size and patterning of the cardiac chambers. At 24 hpf the aberrations in cardiomyocyte number and heart morphology at 24 hpf are moderate. By 48 hpf, the cardiomyocyte number deficiencies became more pronounced with u$du^{-/-}$ hearts containing approximately half the number of cardiomyocytes observed in WT hearts. Furthermore, the percentage of the cardiomyocytes with ventricular identity is reduced in udu mutant embryos at 48 hpf but not at 24 hpf. Based on these findings, we hypothesized that udu is required for the expansion of the cardiomyocyte population, either through reducing apoptosis or by increasing proliferation, and for maintenance of ventricular identity.

3.4.2 Cell death is not responsible for the reduced cardiomyocyte numbers in *udu* mutants.

Zygotic loss of *udu* function has been shown to increase *tumor protein 53* (*tp53)* expression and consequently apoptosis in the 24 hpf zebrafish embryo (Lim et al., 2009; Liu et al., 2007b). Moreover, it was found that reducing *tp53* expression using an antisense morpholino

oligonucleotide (MO) partially restored blood development in *udu* mutants at 48 hpf (Liu et al., 2007b). Embryos homozygous for the *udu^{vu66}* allele also exhibited the increased cell death (Figure 3.9 A, B) and blood deficiencies (Figure 3.2 A, B) observed previously in the zygotic *udu$^{sq1/sq1}$* mutants at 24 hpf (Liu et al., 2007b). Considering that cardiomyocytes and blood share a developmental origin in the lateral plate mesoderm, we hypothesized that the reduction of cardiomyocytes in *udu$^{-/-}$* embryos could share a mechanism with the loss of blood development. Therefore, we tested whether inhibition of TP53-mediated apoptosis would also restore cardiomyocyte number in *udu$^{-/-}$* embryos (Van Vliet et al., 2012).
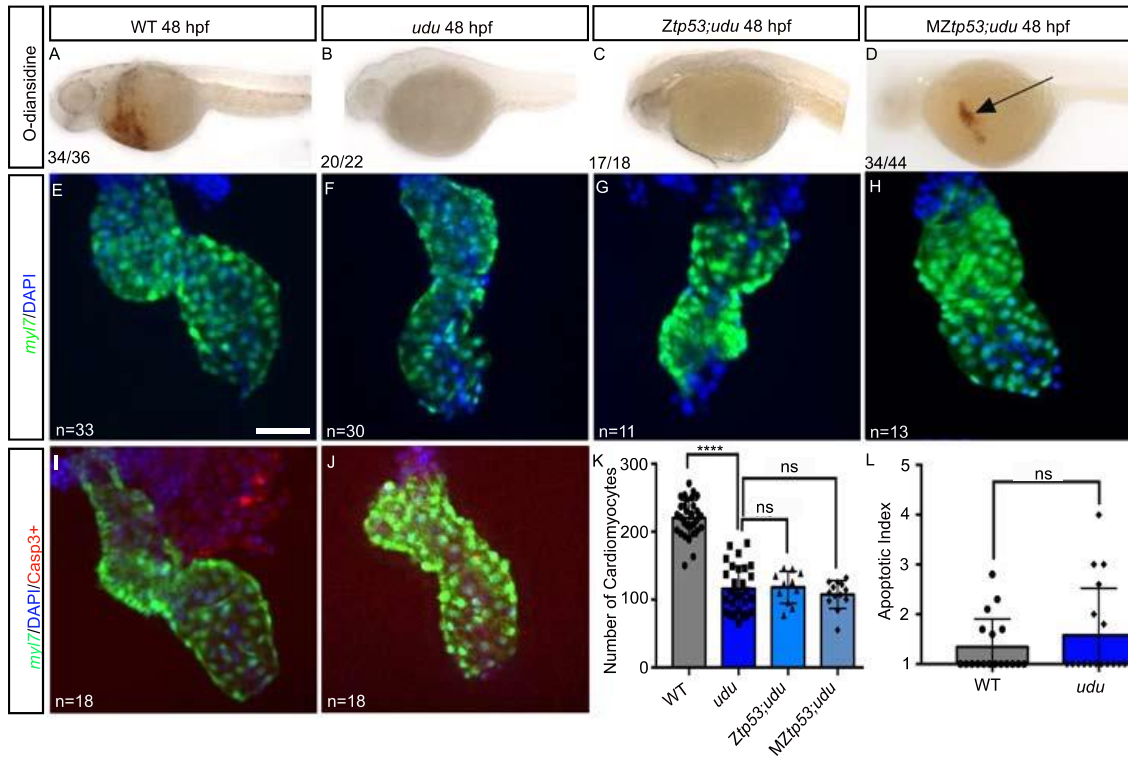
Figure 3.2 The reduced number of cardiomyocytes in *udu* mutant embryos is not caused by *tp53*-dependent apoptosis

Lateral view of showing O-diansidine labeling in WT (A), *udu*^{-/-} (B), Z*tp53*^{-/-};*udu*^{-/-} (C) and MZ*tp53*^{-/-};*udu*^{-/-} (D) 48 hpf zebrafish embryos. Arrow indicates O-diansidine staining in MZ*tp53*^{-/-};*udu*^{-/-} embryos. Dissected hearts labeled with DAPI from Tg(*myl7*:*GFP*) WT (E), *udu*^{-/-} (F), Z*tp53*^{-/-};*udu*^{-/-} (G) and MZ*p53*^{-/-};*udu*^{-/-} (H) embryos at 48 hpf. Quantification of the number of cardiomyocytes in WT, *udu*^{-/-}, Z*tp53*^{-/-};*udu*^{-/-} and *MZtp53*^{-/-};*udu*^{-/-} (K). Casp3 antibody labeling in hearts dissected from 48 hpf WT (I) and *udu* mutant (J) embryos. Graph showing the percentage of Casp3 positive cardiomyocytes in WT and *udu* embryos at 48 hpf (L). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars=SEM. Scale bars represent 50μm.

To this end, we generated $udu^{-/-};tp53^{zdf1/zdf1}$ double mutant zebrafish embryos. To first

establish that we could reproduce the results obtained using a *tp53* MO, we examined these

compound mutants for restoration of blood development by staining for hemoglobin using O-

diansidine at 48 hpf (Berghmans et al., 2005). $udu^{-/-}$ embryos with loss of zygotic *tp53*

expression exhibited no O-diansidine staining (Figure 3.2C) (n=17/18) comparable to that

observed in single $udu^{-/-}$ mutants (Figure 3.2 B). These experiments indicated that zygotic loss of

tp53 was not sufficient to suppress the blood development deficiency in zygotic *udu* mutant

embryos (Figure 3.2 C). However, the *tp53* morpholino studies described above were conducted

with a translation-blocking *p53* morpholino that could, at least partially, block both maternal and

zygotic *tp53* function (Liu et al., 2007b). Hence, we next tested if maternal and zygotic (MZ)

loss of *tp53* could recapitulate the previously described suppression of blood phenotypes in *udu*

mutants (Liu et al., 2007b). We generated $udu^{-/-};MZtp53^{-/-}$ compound mutant embryos by

crossing $udu^{+/-};tp53^{-/-}$ female to $udu^{+/-};tp53^{+/-}$ male fish. O-diansidine staining of the resulting

embryos followed by genotyping demonstrated that the majority (n=34/44) of $udu^{-/-};MZtp53^{-/-}$

embryos displayed a partial restoration of blood development (Figure 3.2 D). This finding

verified the earlier morpholino study, which demonstrated that reduced hematopoiesis in *udu*

mutant embryos is largely dependent on TP53 function (Liu et al., 2007b).

Having confirmed the genetic interaction of *tp53* and *udu* in blood development, we next

asked if loss of zygotic or maternal and zygotic *tp53* expression would increase the number of

cardiomyocytes in $udu^{-/-}$ embryos at 48 hpf. Using the *myl7*:*GFP* transgene to visualize

cardiomyocytes and DAPI to label nuclei, we counted the cardiomyocytes in confocal images of

isolated hearts from WT, *udu, Ztp53;udu* and *MZtp53;udu* mutant embryos (Figure 3.2 E-H,K).

We found that zygotic loss of *tp53* in $udu^{-/-}$ embryos did not result in a significant increase in

cardiomyocyte number (118±7; n=11) (mean ± s.e.m.) compared with $udu^{-/-}$ hearts with functional $tp53$ expression (115±6; n=33) (p=0.84) (Figure 3.2 F,G,K). This result indicates that zygotic loss of $tp53$ is not sufficient to suppress the cardiomyocyte deficiency in udu mutant embryos. Surprisingly, we found that MZ$tp53;udu$ compound mutant embryos also had a similar number of cardiomyocytes (107±6; n= 13) as $udu^{-/-}$ (115±6; n=33) (p=0.41) embryos (Figure 3.2 F,H,K) . This indicates that despite restoring blood development, loss of maternal and zygotic $tp53$ failed to restore cardiomyocyte numbers in udu mutant embryos (Figure 3.2 K). Together, these results demonstrate that the cardiomyocyte deficiency in $udu^{-/-}$ embryos is not the result of $tp53$-dependent apoptosis.

To further corroborate this finding, we assayed the number of apoptotic cells in WT and udu mutant embryos at 48 hpf using Casp3+ immunofluorescent labeling (Figure 3.2 I, J) and found that there was no significant increase in apoptosis of udu mutant cardiomyocytes (0.58±0.22; n=18) relative to WT (0.34±0.13; n=18) (p=0.37) (Figure 2 L). Likewise, TUNEL staining in the embryonic hearts found that the vast majority of both WT (Figure 3.9 C) (n=19/21) and udu mutant hearts (Figure 3.9 D) (n=28/30) contained no apoptotic cells.

Altogether these data indicate that increased cell death is not responsible for the reduced number of cardiomyocytes observed in udu mutant embryos. These findings suggest that the mechanism by which Gon4l increases cardiomyocyte numbers is distinct from its role in hematopoiesis despite their common developmental origin. Furthermore, these results indicate a broader role for Gon4l in the developmental of mesoderm-derived tissues other than repressing cell death.

3.4.3 Cardiomyocyte proliferation is reduced in *udu* mutants

Proliferation is essential for expansion of the cardiomyocyte population following heart tube formation (Leone et al., 2015; Rohr et al., 2006). Based on our finding that the number of cardiomyocytes in $udu^{-/-}$ embryos is static between 24 and 48 hpf (Figure 3.1 Q), we decided to examine whether the proliferation rate of cardiomyocytes is affected in $udu^{-/-}$ embryos. To this end, we employed EdU incorporation in 36 hpf embryos to mark cardiomyocytes that progress through S-phase of the cell cycle (Figure 3.3 A) (Salic and Mitchison, 2008). We labeled cardiomyocyte nuclei with Mef2 antibody. We then obtained confocal images of the hearts and counted the number of double EdU and Mef2 positive cells.

We found that cardiomyocyte proliferative index - the percent of cardiomyocytes that incorporated EdU - was significantly reduced in $udu^{-/-}$ embryos (Figure 3.3 C, D) (0.59±0.2; n=18) compared to WT (2.6±0.35; n=13) (Figure 3 , D white arrow) suggesting that *udu-/-* cardiomyocytes have a reduced capacity to progress through S-phase of the cell cycle (p<0.0001). This reduction in proliferation appears to also affect non-cardiomyocytes in the heart with the presumed endocardium of WT hearts (Figure 3 B, green arrow) having EdU+ cells while udu$^{-/-}$ hearts contained fewer EdU+ cells in non-cardiomyocytes as well (Figre 3.3 C).
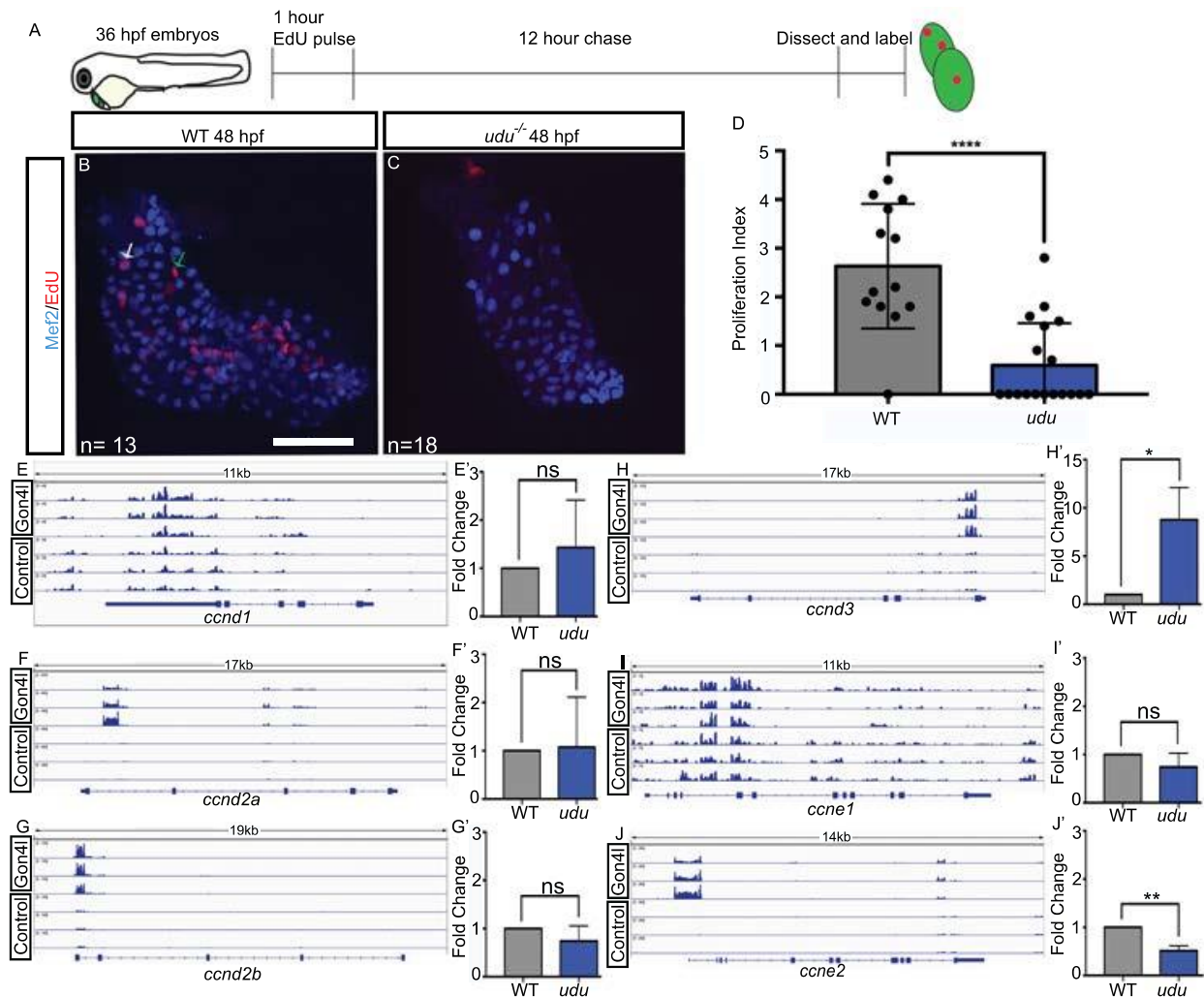
Figure 3.3 EdU incorporation is reduced in the hearts of *udu* mutant embryos

Experimental workflow for EdU labeling (A). EdU labeling (red) in 48 hpf WT hearts (B) and

udu^{-/-} hearts (C) with cardiomyocytes labeled by Mef2 (blue). Arrows showing EdU+

cardiomyocyte (white) and non-cardiomyocyte (green). Graph showing the proliferative index

for EdU in WT (grey) and *udu*^{-/-} (blue) cardiomyocytes (D). DamID-seq Genome browser tracks

of Gon4l-Dam and GFP Control-Dam at *ccnd1* (E), *ccnd2a* (F), *ccnd2b* (G), *ccnd3* (H), *ccne1*

(I), and *ccne2* (J) loci. qRT-PCR for *ccnd1* (E'), *ccnd2a* (F'), *ccnd2b* (G'), *ccnd3* (H'), *ccne1*

(I'), and *ccne2* (J') performed on RNA extracted from hearts isolated from 36 hpf WT (gray) and

udu^{-/-} (blue) embryos. Results are shown as fold difference in gene expression in *udu* compared

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to WT and results were standardized to *gapdh* expression. *p<0.05, **p<0.01, ***p<0.001,
****p<0.0001, error bars=SEM. Scale bar represents 50μm.

These EdU results indicate that cardiomyocyte proliferation is reduced in *udu* mutant embryos, implicating a failure of cell cycle progression. Previous studies found that the expression of cell cycle regulator *Cyclin D (Cnnd3)* was reduced in multipotent progenitors isolated from *Gon4l* mutant mice (Barr et al., 2017). To test if this defect is conserved in zebrafish hearts, we measured the expression levels of the D and E type cyclins in *udu* mutant embryos: *ccnd1, ccnd2a, ccnd2b, ccnd3, cnne1,* and *cnne2.* We isolated RNA from hearts dissected at 36 hpf from *udu* mutant and WT embryos. Using qRT-PCR analysis we found that levels of *cnnd1, ccnd2a, ccnd2b,* and *ccne1* (Figure 3.3 E', F', G', I') were unchanged at this stage (p>0.05). However, two cyclins were differentially expressed in the *udu* mutant hearts. Expression level of the early G1-stage cyclin *ccnd3* was significantly increased in *udu* mutant hearts (Figure 3.3 H') compared to WT (p=0.016). We also found that the expression of *cnne2* (Figure 3 J') was significantly reduced in the *udu* mutant hearts relative to WT (p=0.001) (Figure 3 F). *cnne2* expression peaks at the G1/S phase transition and is required for entry into S-phase , thus further corroborating our results from EdU experiments.

We next probed whether D and E type *cyclins* could be direct targets of Gon4l. We analyzed previously published zebrafish DNA adenine methyltransferase identification with sequencing (DamID-seq) data, a which uses methylation from a fusion protein construct to identify regions of protein-genome association, to ask if Gon4l was associated with the regulatory regions of any of these genes (Steensel, 2001; Williams et al., 2018). Our analysis found that by tailbud stage, Gon4l association was enriched at regions near *ccnd2a, ccnd2b, ccnd3*, and *cnne2* (Figure 3.3 E-J). These data suggest that Gon4l may directly regulate the expressions of these *cyclins*. One caveat is that the DamID-Seq data analyzed was collected at tailbud stage and is from whole gastrulae and may not accurately reflect Gon4l association at

later stages. Nevertheless, these DamID-Seq data together with our qRT-PCR and EdU results indicate a potential role for Gon4l in mediating progression into S-phase through regulating expression of G1/S phase cyclins.

3.4.4 Expression of nkx2.5 and nkx2.7 is reduced in udu mutants following heart tube formation

Studies in zebrafish have defined two distinct phases of cardiomyocyte development. First, the primary heart field is specified in bilateral regions of lateral plate mesoderm (LPM) during early segmentation. Then, after heart tube formation, the second heart field extends the two poles of the heart tube and contributes to the development of the two cardiac chambers (Buckingham et al., 2005; Cai, 2003; De La Cruz, 1977). The transcription factors Nkx2.5 and Nkx2.7 play critical roles in the specification of both the primary and second heart fields and are necessary for maintenance of ventricular identity in zebrafish embryos (George et al., 2015; Targoff et al., 2013; Targoff et al., 2008).
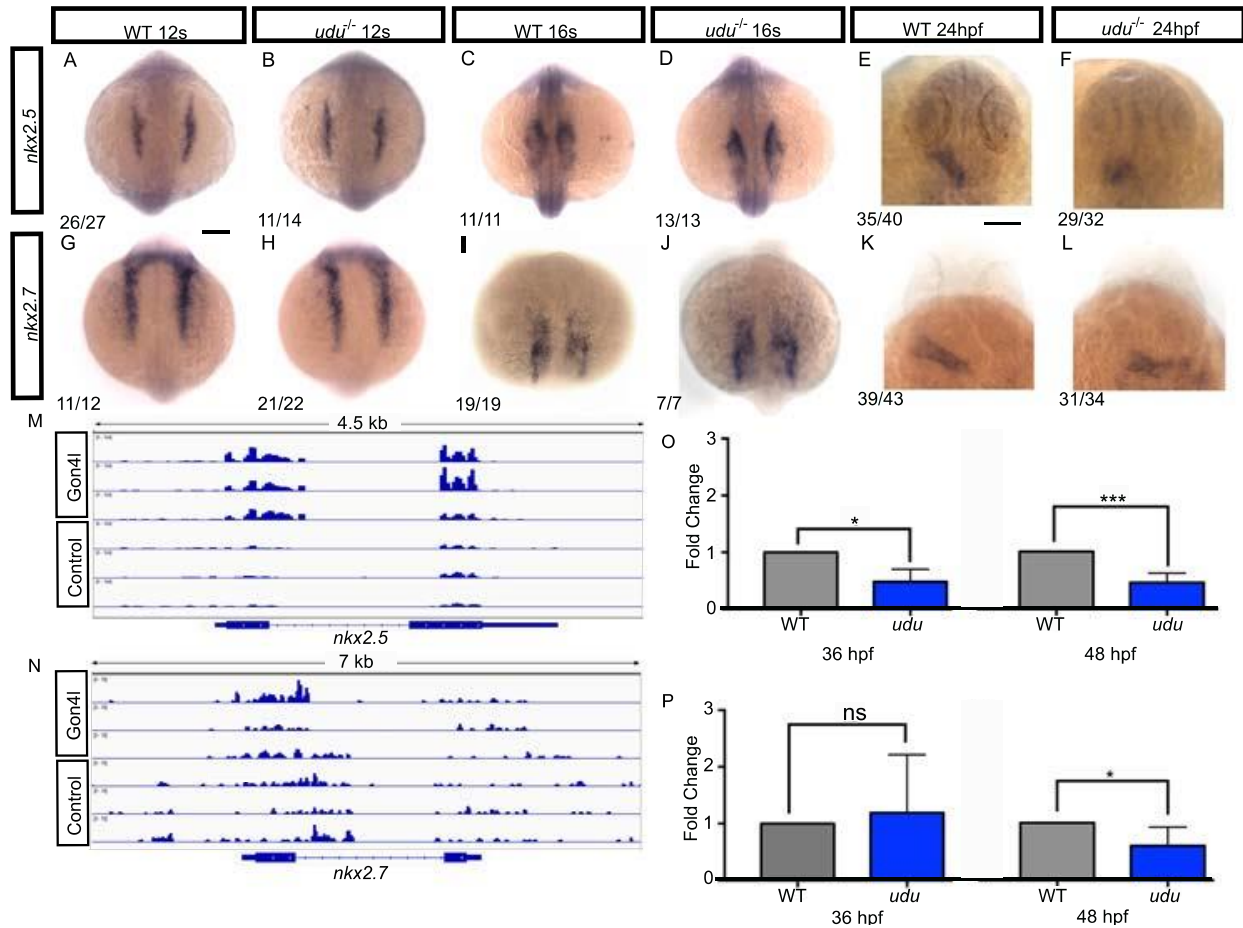
Figure 3.4 Expression of *nkx2.5* and *nkx2.7* is initiated normally in the nascent cardiomyocytes of *udu* mutants but becomes reduced following heart tube formation

Dorsal view of whole mount *in situ* hybridized embryos showing *nkx2.5* (A-F) and *nkx2.7* (G-L) expressions and in WT (A, C, E, G, I, K) and *udu*$^{-/-}$ (B, D, F, H, J, L) embryos at 12s (A, B, G, H), 16s (C, D, I, J) and 24 hpf (E, F, K, L) stages. DamID-seq Genome browser tracks of Gon4l-Dam and GFP Control-Dam at *nkx2.5* (M) and *nkx2.7* loci. Graph of qRT-PCR results performed on RNA extracted from hearts isolated from 36 hpf and 48 hpf WT and *udu*$^{-/-}$ embryos for *nkx2.5* (O) and *nkx2.7* (P) expression; results were standardized to *gapdh* expression. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars=SEM. Genome browser tracks of Gon4l-DAM and GFP Control-Dam at *nkx2.5* (M) and *nkx2.7* (N) locus. Scale bars represent 50μm.

Given that previous studies in mouse embryonic stem cells suggest that *nkx2.5* expression can be regulated by the Gon4l binding partner Yy1 (Gregoire et al., 2013; Lu et al., 2011), and the established role of Nkx2.5 in patterning heart chambers and maintaining ventricular fate (Gregoire et al., 2013; Lu et al., 2011; Targoff et al., 2013; Targoff et al., 2008), we next examined the expression domains of *nkx2.5* (Figure 3 A-E) and *nkx2.7* (Figure 3.4 G-L), We performed WISH in *udu* mutants and WT embryos during late segmentation stages through cardiac heart tube formation. During CPC specification at 12-somite stage, the majority of both WT (n= 26/27) and *udu*$^{-/-}$ (n=11/14) embryos presented normal bilateral expression domains of *nkx2.5* in the ALPM (Figure 3.4 A,B). Likewise at 16-somite stage, *nkx2.5* was expressed in comparable domains in WT (n=11/11) and *udu* mutant embryos (n=13/13) (Figure 3.4 C,D). At 12-somite stage, *nkx2.7* expression domains in *udu* mutant (Figure 3.4 H) (n=21/22) were similar to WT (Figure 4 G) (n=11/12) embryos. As well, at 16-somite stage *udu* mutant embryos (Figure 4 J) (n=7/7) and WT (Figure 3.4 I) (n=19/19) had equivalent expression domains of *nkx2.7*.

However, by 24 hpf, during cardiac chamber patterning, *nkx2.5* (Figure 3.4 E, F) and *nkx2.7* (Figure 3.4 K, L) had misshaped expression domains in *udu* mutant embryos compared to WT. To assess whether the differences in expression domains could reflect changes in gene expression levels, we performed qRT-PCR on RNA isolated from hearts of WT and *udu* mutant embryos at 36 hpf and 48 hpf. On hearts from 36 hpf embryos, qRT-PCR revealed that expression of *nkx2.5* (Figure 3 O)(p=0.02), but not *nkx2.7* (p=0.72) (Figure 3.4 P) was reduced in hearts isolated from embryos compared to WT. qRT-PCR analysis on RNA isolated from hearts found that at 48 hpf expression levels of both *nkx2.5* (p=0.0006) and *nkx2.7* (p=0.05) were significantly decreased (Figure 3.4 O, P).

We analyzed zebrafish DamID-Seq datasets to ask if either of these genes could be a direct target of Gon4l. The analysis showed an enrichment of Gon4l association near *nkx2.5* (Figure 3.4 M) but not *nkx2.7* (Figure 3.4 N). Previously, Gon4l could be secondarily linked to Nkx2.5 through interactions with Yy1, but these DamID-Seq data provide the evidence that Gon4l could directly regulate expression of the cardiac transcription factor. From these experiments we conclude that Gon4l is involved in maintaining *nkx2.5* expression during heart tube formation and cardiac chamber emergence.

3.4.5 *udu* genetically interacts with *nkx2.5* maintain ventricular identity

Nkx2.5 is a key regulator of ventricular fate (Targoff et al., 2013; Targoff et al., 2008; Tu et al., 2009). Considering the reduced expression of *nkx2.5* in *udu* mutant embryos post-heart tube formation (Figure 3.4 O), the reduced ventricular identity observed in these embryos, and expansion of atrial cells (Figure 3.1 P, R), we wanted to interrogate whether *udu* genetically interacted with *nkx2.5*.

To test this, we characterized cardiac chamber patterning in the progeny of $nkx2.5^{+/-}$;$udu^{+/-}$ double heterozygotes using antibodies that label the atrium (S46) and the entire myocardium (MF20) and Mef2 to label cardiomyocyte nuclei (Figure 5 A). We noted that compared to both $udu^{-/-}$ (Figure 3.5 B) and $nkx2.5^{-/-}$ (Figure 3.5 C) *nkx2.5;udu* double mutant embryos appeared to have a more severe loss of ventricular identity than either single mutant.
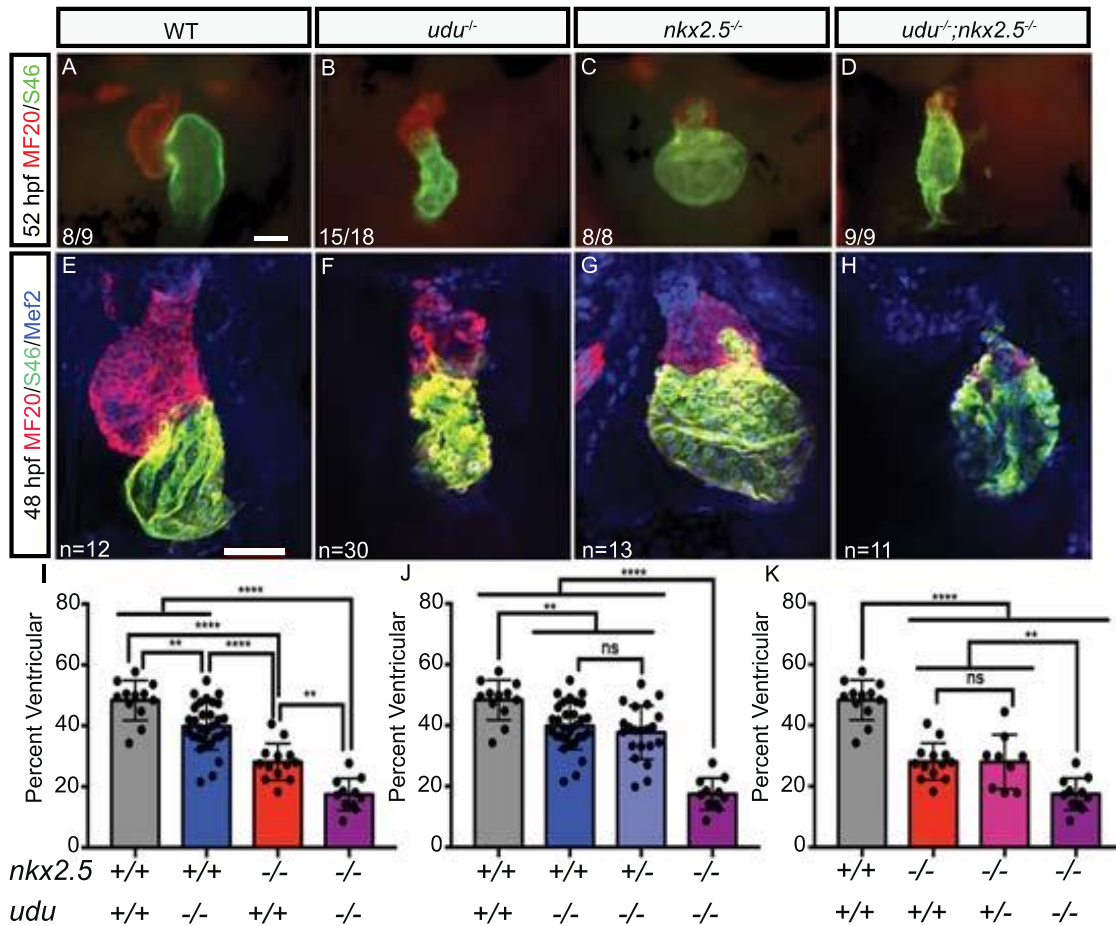
Figure 3.5 *udu* genetically interacts with *nkx2.5* to maintain ventricular identity

Ventral view of WT (A), *udu*^{-/-} (B), *nkx2.5*^{-/-} (C), and *udu*^{-/-};*nkx2.5*^{-/-}(D) zebrafish hearts at 52 hpf with immunofluorescence showing the myocardium (MF20, red) and atrium (S46, green). Ventral view of WT (E), *udu*^{-/-} (F), *nkx2.5*^{-/-} (G), and *udu*^{-/-};*nkx2.5*^{-/-}(H) showing the myocardium (MF20, red) and atrium (S46, green) and cardiomyocyte nuclei (Mef2, blue) at 48 hpf. Quantification of the percent of total cardiomyocytes expressing ventricular markers (F, G, H). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, error bars=SEM. Scale bar represents 50μm.

In order to better quantify the genetic interaction between *nkx2.5* and *udu*, we employed Mef2 antibody labeling together with MF20 and S26 antibody labeling to enable us to count the number of cardiomyocytes with atrial and ventricular identities (Figure 3.5 E). We found that the percentage of cardiomyocytes with ventricular identity is significantly reduced in both *udu* (40±1.4, n=30) (p=0.036) (Figure 3.5 F, I) and *nkx2.5* (28±1.7, n=13)(p<0.0001) (Figure 3.5 G, I) single mutant embryos relative to WT (48±1.8, n=12) (Figure 3.5 A, I). We then examined *nkx2.5; udu* double mutant hearts, and we observed an almost complete loss of ventricular identity with domains of atrial cells being expressed throughout the entire heart (Figure 3.5 H). We quantified atrial and ventricular cardiomyocytes we determined that *nkx2.5; udu* (Figure 3.5 H) (17±1.6, n=11) double mutant embryo hearts had a more severe reduction in the percentage of cardiomyocytes expressing ventricular identity compared to either *udu* (40±1.4, n=30) (p<0.0001) or *nkx2.5* (28±1.7, n=13)(p=0.002) single mutants (Figure 3.5 I).

Heterozygous loss of neither *nkx2.5* nor *udu* has an effect on cardiac chamber patterning in WT or *nkx2.5* or *udu* mutant backgrounds (Figure 3.5 J, K and Figure 3.10 B-H, J). This suggests that the interaction is not dose-dependent and that loss of a single copy of *nkx2.5* or *udu* is not sufficient to perturb cardiac chamber patterning. Taken together, these results intimate that *nkx2.5* and *udu* genetically interact during maintenance of ventricular identity.

3.4.6 Overexpression of *nkx2.5* is not sufficient to suppress *udu* heart defects

Although our results indicate that *nkx2.5* and *udu* genetically interact in maintenance of ventricular identity, these studies do not address whether the effect of the loss of *udu* expression on ventricular fate is solely due to interactions with *nkx2.5*. To this end, we tested if overexpressing *nkx2.5* in *udu* mutant embryos was sufficient to suppress ventricular fate defects.

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We generated *udu*^{-/-};Tg(*hsp70l:nkx2.5*-eGFP)^{+/-} embryos using the previously validated Tg(*hsp70l:nkx2.5-e*GFP) transgenic line (George et al., 2015). These embryos ubiquitously express *nkx2.5-eGFP* upon heat shock (George et al., 2015), which enabled us to determine if overexpressing *nkx2.5* was sufficient to restore proper maintenance of ventricular fate in *udu*^{-/-} embryos. *nkx2.5* expression was induced via heat shock at 21-somite stage (Figure 3.6 A). GFP expression was visible within an hour of heat shock and was used to select embryos carrying the transgene (George et al., 2015). We analyzed cardiac chamber patterning and cardiomyocyte numbers using MF20, S46 and Mef2 immunostaining in *udu*^{-/-} embryos with and without Tg(*hsp70l:nkx2.5*-eGFP) at 48 hpf (Figure 6 B-E).
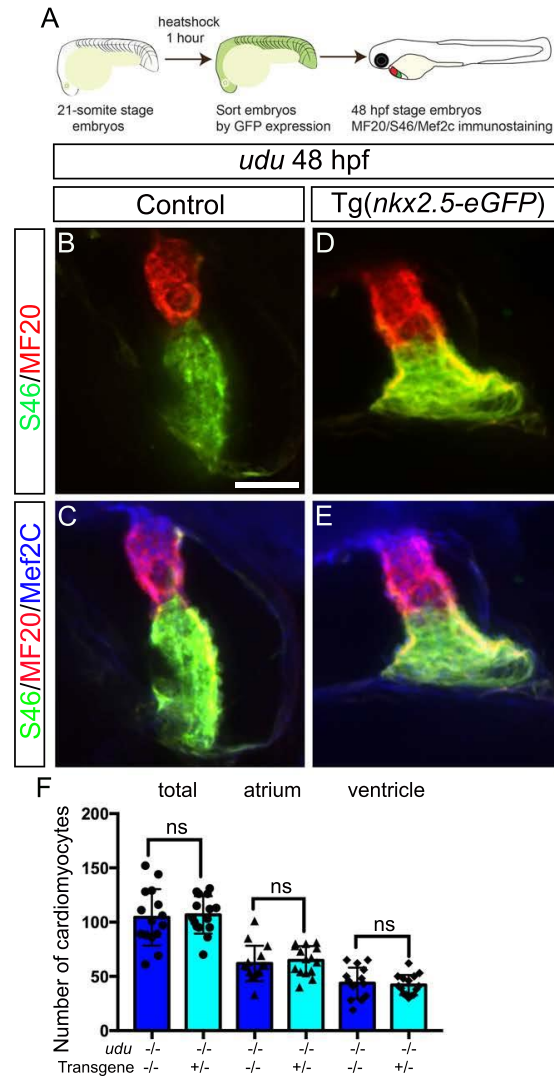
Figure 2.6 Ectopic *nkx2.5* expression is not sufficient to restore ventricular identity in *udu* mutant embryos

Experimental workflow (A). Immunofluorescent labeling with S46 (green) labeling the atrium, MF20 (red) labeling the myocardium 48 hpf $udu^{-/-}$ embryos (B) and merged with Mef2 images (C) and in $udu^{-/-}$;Tg(*hsp70l:nkx2.5-eGFP*) mutant embryos (D, E). Graph showing the total cardiomyocytes, number of cells in the atrium and ventricle in *udu* mutant embryos with and without Tg(*hsp70l:nkx2.5-eGFP)* (F). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, error bars=SEM. Scale bar represents 50μm.

We tested the affects of the ectopic nkx2.5 expression in *udu* mutant embryos. We observed that *udu*$^{-/-}$ embryos carrying the Tg(*hsp70l:nkx2.5*-eGFP) that where subjected to heat shock (Figure 3.6 D, E) did not exhibit a significant decrease an increase in proportion of cardiomyocytes with ventricular identity (43±2.6, n=15) (Figure 3.6 E) relative to *udu*$^{-/-}$ siblings without the transgene (Figure 3.6 B, C) (41±1.6, n=14) (p=0.51) after heatshock (Figure 3.6 E). Likewise, the overall number of cardiomyocytes was unchanged in *udu*$^{-/-}$; Tg(*hsp70l:nkx2.5*-eGFP) (104.4±6.7, n=15) embryos compared to *udu* mutant sibling control embryos (106.7±4.4, n=15) (p=0.78) (Figure 3.6 F). This result indicates that exogenous *nkx2.5* expression is not sufficient to increase the number of cardiomyocytes (ventricular or otherwise) in *udu*$^{-/-}$ embryos.

The inability of exogenous *nkx2.5-eGFP* expression to suppress the expansion of atrial identity in *udu*$^{-/-}$ embryos indicates that Gon4l does not maintain ventricular identity solely through modulation of *nkx2.5* expression. These results together with the reduced expression of *nkx2.*5 in *udu* mutant hearts and the DamID-seq analysis suggesting that Gon4l associates with *nkx2.5* genomic locus point to a few possibilities for how Gon4l is acting during maintenance of ventricular identity. Gon4l could potentially regulate the *nkx2.5* chamber-patterning cascade at multiple levels, act in a parallel pathway, or combination of both of these possibilities (Figure 7).

3.5 Discussion

This study establishes a novel role for the chromatin factor Udu/Gon4l in heart development (Figure 7). Gon4l promotes cardiomyocyte proliferation and maintains ventricular identity during zebrafish embryogenesis, functioning independently of TP53-induced apoptosis. In zebrafish embryos lacking zygotic *udu* function, the initial formation and patterning of the heart tube is largely intact. During later stages of heart development, however, Gon4l is necessary for expansion of the cardiomyocyte pool by promoting their proliferation. Furthermore, *udu* genetically interacts with *nkx2.5* to maintain the expression of ventricular fate markers. Our studies, therefore, identify a chromatin factor with multiple novel roles in heart development.

This work highlights the role of Gon4l in cell cycle regulation during cardiomyocyte population expansion. Previous studies established a role for Gon4l in cell-cycle regulation in somitogenesis and blood development (Barr et al., 2017; Hammerschimidt et al., 1996; Lim et al., 2009; Liu et al., 2007b; Lu et al., 2011; Lu et al., 2010), but its contributions to heart development were not previously examined. Many of our findings agree with the roles previously identified in zebrafish and mouse studies in these other contexts. Namely, that Gon4l regulates both cell proliferation and differentiation. However, key differences from preceding work emerge in our data. Our genetic interaction studies with *tp53* found that the reduction in cardiomyocyte numbers is TP53- and apoptosis independent. This result contrasts with the *udu* blood deficiency phenotype, which was partially suppressed by reduction of *tp53* expression using antisense morpholinos (Lim et al., 2009; Liu et al., 2007b) and genetic maternal-zygotic loss of function (Figure 2). Our data, therefore, indicate that the cardiac defects arise through a separate mechanism than the hematopoietic defects observed in the *udu* mutant embryos.

Figure 2.7 Roles of Gon4l in heart development

This study establishes a novel role for the chromatin factor Udu/Gon4l in heart development (Figure 7). In zebrafish embryos lacking zygotic *udu* function, the initial formation and patterning of the heart tube is largely intact. During later stages of heart development, however, Gon4l is necessary for expansion of the cardiomyocyte pool and maintenance of ventricular identity. We presented several lines of evidence that Gon4l promotes cardiomyocyte proliferation, functioning independently of TP53-induced apoptosis. Furthermore, *udu* genetically interacts with *nkx2.5* to maintain the expression of ventricular fate markers. Our studies, therefore, identify a chromatin factor with multiple novel roles in heart development.

Previous studies established a role for Gon4l in cell-cycle regulation in somitogenesis and blood development in zebrafish and mouse (Barr et al., 2017; Hammerschimidt et al., 1996; Lim et al., 2009; Liu et al., 2007b; Lu et al., 2011; Lu et al., 2010), but its contributions to heart development were not previously examined. Many of our findings agree with the roles previously identified in zebrafish and mouse studies in these other contexts. Namely, that Gon4l regulates both cell proliferation and differentiation. However, key differences from preceding work emerge in our data. Our genetic interaction studies with *tp53* found that the reduction in cardiomyocyte numbers is TP53- and apoptosis independent. This result contrasts with the *udu* blood deficiency phenotype, which was partially suppressed by reduction of *tp53* expression using antisense morpholinos (Lim et al., 2009; Liu et al., 2007b) and genetic maternal-zygotic loss of function (Figure 2). Our data, therefore, indicate that the cardiac defects arise through a separate mechanism than the hematopoietic defects observed in the *udu* mutant embryos.

Our data point to regulation of proliferation by Udu/Gon4l as this distinct mechanism. First, we found that cardiomyocyte proliferation is reduced in $udu^{-/-}$ embryos at 36 hpf, likely due to a failure to progress through S-phase of the cell cycle (Figure 7). Gon4l has been linked to cell

cycle regulation in both mouse and zebrafish (Barr et al., 2017; Lim et al., 2009), but there has been disagreement regarding which phase or phases of the cell cycle are disrupted upon loss of Gon4l function.

In our study we found that expression of *cnne2*, a gene necessary for the G1/S phase transition (Caldon, 2010), was downregulated in a heart specific manner in $udu^{-/-}$ embryos at 36 hpf (Figure 3). Studies of multipotent blood progenitors in *Gon4l* mutant mouse found that *Cnnd* homologues are down regulated during B-cell development while *Cnne* was normally expressed (Barr et al., 2017). The main difference in our results is that *cnnd3* is overexpressed relative to WT levels in hearts of *udu* mutant embryos while *cnne2* expression was downregulated. Altogether our results, in combination with previous studies, support an essential role for Gon4l in regulating G1/S check-point of the cell cycle (Barr et al., 2017; Lim et al., 2009). DNA adenine methyltransferase identification (DamID-seq), a technique that detects protein-chromatin interactions (de Groote et al., 2012; Steensel, 2001), indicates that Gon4l is associated with the promoters of *ccnd2a*, *ccnd2b*, *ccnd3*, and *ccne2* during early zebrafish development (Williams et al., 2018). There are important caveats to keep in mind with the DamID-seq data. Namely, that the experiments were performed at tailbud stage, which is long before the phenotypes described herein arise. Additionally, DamID-seq cannot determine if a complex containing Gon4l binds at a given locus, it provides a read out of proximity to a region. Nevertheless, this DamID-seq data suggests that these *cyclins* could be direct targets of Gon4l regulation.

Our studies also establish a requirement for Gon4l in the maintenance of ventricular identity. In *udu* mutant embryos, there is a decrease in the percentage of cardiomyocytes with ventricular identity compared to WT embryos at 48 hpf. We find that *nkx2.5* and *udu* genetically interact in maintaining ventricular identity, and Gon4l is necessary for the maintenance of *nkx2.5*

expression after heart tube formation. DamID-seq data indicates that Gon4l is associated with the *nkx2.5* promoter during early zebrafish development (Williams et al., 2018). Previously, Gon4l could be secondarily linked to Nkx2.5 through interactions with Yy1, but these DamID-Seq data provide the evidence that Gon4l could directly regulate expression of the cardiac transcription factor.

The discovery that *nkx2.5* genetically interacts with *udu*, but that exogenous expression of *nkx2.5* using the Tg(*hsp70l:nkx2.5*-eGFP) line is unable to suppress the reduction of ventricular fate opens a few possible explanations for how Gon4l affects cardiac chamber identity. In the simplest model, *nkx2.5* is the main target of Gon4l, but it is regulated during many stages of development and additional pulses of ectopic *nkx2.5* would be needed to restore cardiac chamber patterning. A more complex explanation is that Nkx2.5 sits atop a large cascade of cardiac genes including *irx4, myl7*, and *hand1* (Harvey, 2002). Given Gon4l's role as a chromatin factor (Lu et al., 2011), it is possible that Gon4l regulates both Nkx2.5 and its downstream gene cascade (Figure 7). In an alternative model, other transcription factors such as Gata4 and Hand2 (Brown et al., 2004; Yelon, 2000) work in parallel and interacting pathways to Nkx2.5 in heart development and could potentially be regulated by Gon4l. Or it is possible to Gon4l regulates maintenance of ventricular identity through Nkx2.5 and its downstream targets as well as through parallel pathways.

In total, these results highlight the role that epigenetic regulators play in modulating the cardiac chamber identity and open intriguing hypotheses regarding the role of Gon4l in this process.

In conclusion, defining the precise roles of chromatin factors during cardiac development is an on-going process. This study elucidates hereunto unknown and essential roles for Gon4l during the processes of heart development after formation of the heart tube. Our results suggest

that Gon4l both promotes cell proliferation and regulates the expression of cardiac transcription factors to maintain ventricular identity. These findings describe a novel regulator of heart development and provide insights into heart patterning and cardiomyocyte proliferation that offer new targets in understanding congenital heart defects.

3.6 Supplemental Information
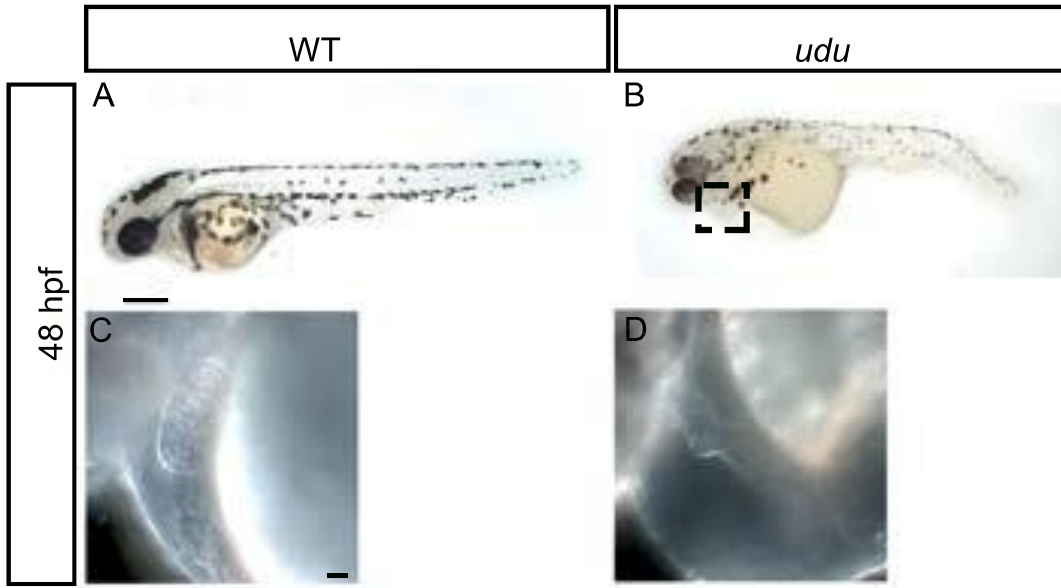
Figure 2.8 *udu* mutant embryos exhibit cardiac defects

Lateral view of *udu*-/- and WT embryos (A,B) and their heart region (C,D) at 48 hpf.
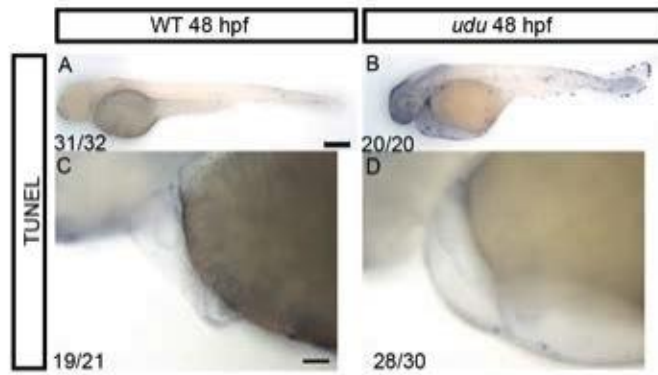
Figure 2.9 Apoptosis level is unchanged in the hearts of *udu* mutant embryos

TUNEL staining performed in WT (A,C) and *udu*$^{-/-}$ (B,D) whole embryo (A,B) and heart (C,D)

at 48 hpf. Scale bars represent 50μm.
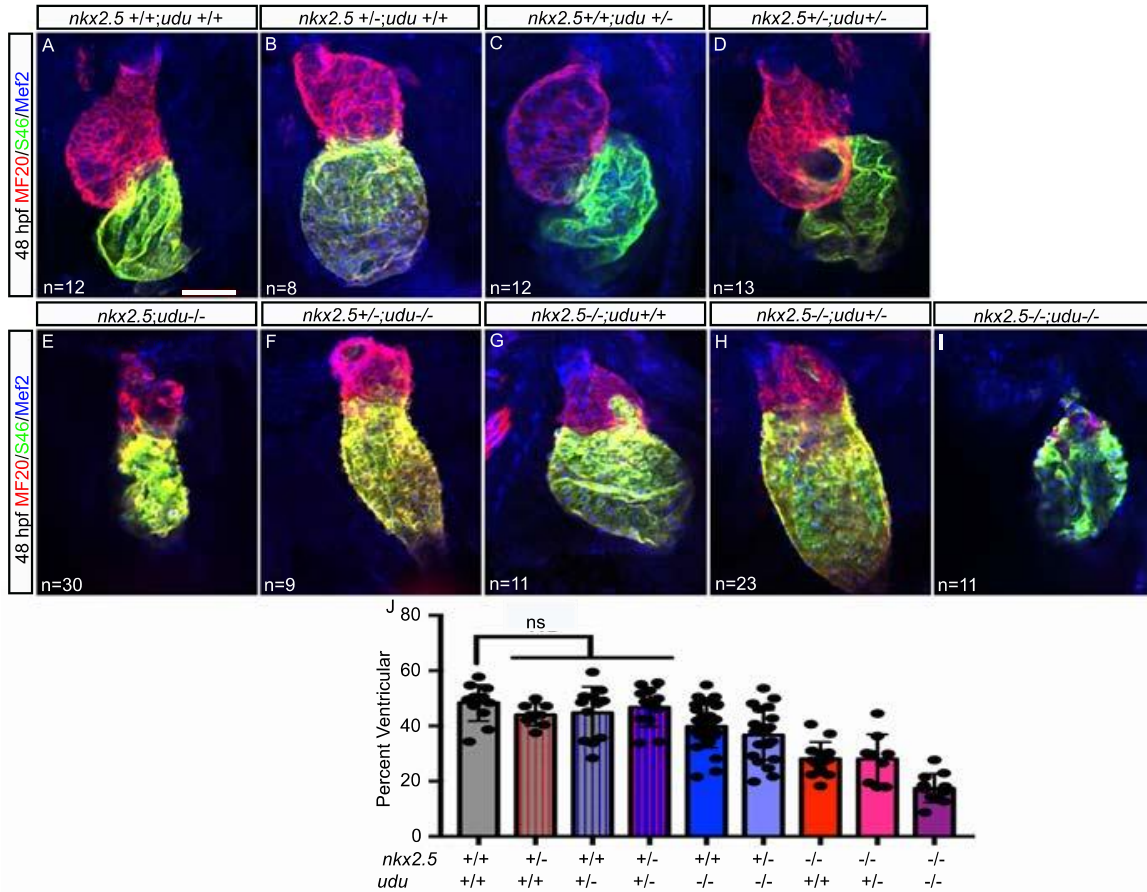
Figure 2.10 Genetic interaction between *nkx2.5* and *udu* is not dose-dependent in maintenance of ventricular identity

WT heart labeled with MF20, S46, and Mef2 (A). Heterozygous hearts for *nkx2.5* (B), *udu* (C), and both (D). *udu* mutant heart (E) and *udu* mutant heart heterozygous for *nkx2.5* (F). *nkx2.5* mutant heart (shown in main) (G) and *nkx2.5* mutant heart heterozygous for *udu* (H). *nkx2.5;udu* double mutant heart (I). Quantification of the percent of cardiomyocytes with ventricular identity (J). A, E, G, and F shown in main figure 3.5. Scale bar represents 50μm.

Chapter 4: Discussion

4.1 Perspective

Congenital heart defects (CHDs) impart massive costs both in human life and monetarily. The causes of CHDs are varied including both environmental and genetic contributions. The genetic defects can arise during many stages of embryonic development and lead to diverse phenotypes in CHDs (Pierpont et al., 2007; Sadler, 2017). Therefore, it is imperative to more fully understand the process of heart development to improve diagnostics and treatments. Epigenetic modifications likely underpin the regulation of numerous regulatory networks during cardiogenesis, and therefore, provide an avenue by which to dissect the pathways that control heart development. In this dissertation, we define the maternal and zygotic roles of Gon4l in heart development (Figure 4.1). We demonstrate that maternal *udu* expression is needed to pattern the mesoderm and initiate expression of cardiac transcription factors. We also elucidate zygotic functions of Gon4l in regulating cardiomyocyte proliferation and maintaining ventricular identity. Together, these results indicate that Gon4l mediates many aspects of cardiac development and that its expression is essential in vertebrate heart formation.

Gon4l is a highly conserved chromatin factor with homologues present from land plants to humans (Brownfield et al., 2009; Wang et al., 2004). In vertebrates, Gon4l has established functions in hematopoiesis, somitogenesis, apoptosis, and cell-cycle regulation (Barr et al., 2017; Lim et al., 2009; Liu et al., 2007b; Lu et al., 2011; Lu et al., 2010; Williams et al., 2018). Despite the ubiquitous maternal expression of *udu*, other research groups have not explored the early phenotypes in embryos lacking both maternal and zygotic expression of *udu*.
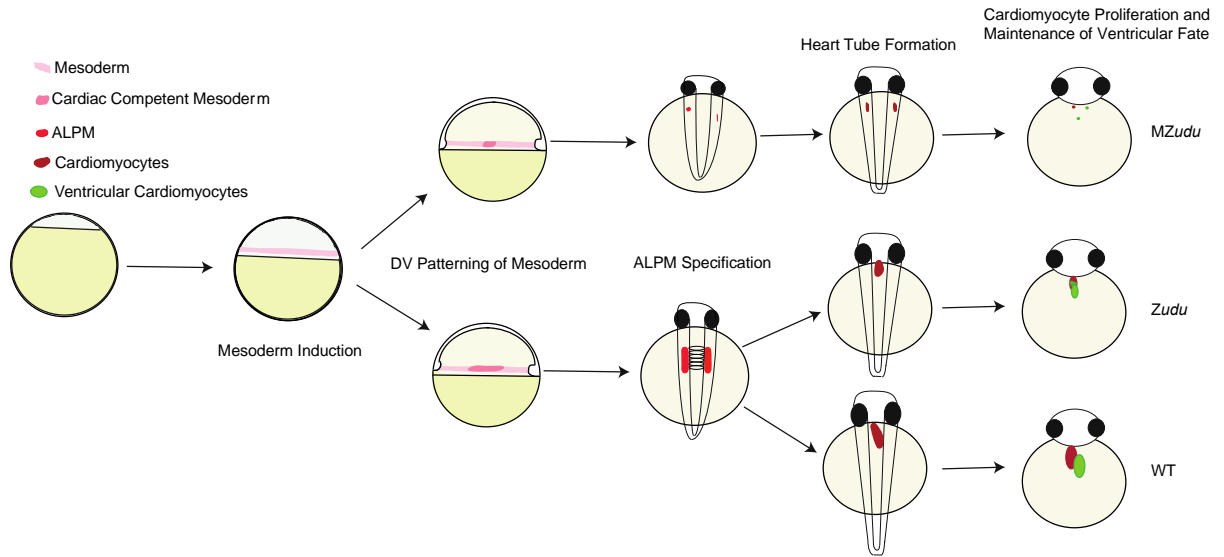
Figure 4.1 Schematic representation of the phenotypes associated with zygotic and maternal-zygotic removal of *udu* expression

4.2 Gon4l in germ layer induction and mesoderm patterning

In Chapter 2, we investigate the phenotypes of MZ*udu* mutant embryos and define the functions of Gon4l from germ layer formation through cardiac chamber patterning. We show that mesoderm is induced in MZ*udu* mutant zebrafish embryos, but the expression of the genes that confer DV patterning upon the embryo is perturbed. By the end of gastrulation, MZ*udu*$^{-/-}$ embryos present defects in mesoderm-derived tissues. Our RNA-seq studies elucidated broad disruption of the transcriptome in MZ*udu*$^{-/-}$ embryos. By tailbud stage, more than 10% of the transcriptome is differentially expressed in MZ*udu*$^{-/-}$ mutant embryos with genes expression being both up- and down-regulated. Gene ontology (GO) analysis identified an enrichment of differentially expressed genes in terms related to DNA-binding and germ layer development. More specifically, we demonstrate that genes involved in mesoderm development are also broadly differentially expressed. We observed that the lateral plate mesoderm, comprising the anterior (ALPM) and ventral (VLPM), was among the most affected of the mesoderm sub-types. We demonstrated that the heart, the main organ arising from the ALPM, is almost absent at 24 hpf and 48 hpf in MZ*udu* mutant embryos.

Cell autonomy experiments we performed indicated that the severe cardiac phentoypes observed in MZ*udu* mutant embryos have both cell-autonomous and non-cell-autonomous contributions. This result provides additional support for the notion that Gon4l regulates heart development through many genetic pathways and mechanisms. We attempted to test many of the potential mechanisms of Gon4l in heart development, but we were unable to conclusively suppress any of the cardiac defects through rescue experiments.

The non-cell autonomous role presumably arises during the initial DV patterning of the mesoderm, which acts to define the cardiomyocyte competent region of the mesoderm. Retinoic

acid (RA) signaling is a potential non-cell autonomous regulator of heart development that has aberrant expression in MZ*udu* mutant embryos. RA signaling limits the size of the cardiac competent mesoderm, and when components of the pathway are overexpressed, as in MZ*udu* mutant embryos, heart size is reduced (Begermann, 2001; Liu and Stainier, 2012). We tested if inhibiting retinoic acid signaling using diethlaminobenzaldehyde (DEAB) (Le et al., 2012) could partially restore heart development and axis extension in the MZ*udu* mutant embryos. Adding DEAB to MZ*udu*$^{-/-}$ embryos at various stages of gastrulation and in different doses neither restored axis extension nor heart development (Williams and Budine, unpublished). This result strongly suggests that retinoic acid signaling is not largely responsible for the reduced heart size in the MZ*udu* mutant embryos. However, these experiments cannot eliminate RA signaling from playing more subtle roles in the defects observed in MZ*udu* mutant embryos or the inhibition requiring careful temporal or dose titrations. More thorough titrations of DEAB doses and timing of application will be needed as well testing RA signaling in conjunction with other pathways to fully absolve it from causes phenotypes in MZ*udu*$^{-/-}$ embryos.

Another secreted pathway of interest in germ layer patterning and heart development is the Wnt pathway. Wnt signaling was disrupted in the MZ*udu*$^{-/-}$ embryos at gastrulation stages according to both WISH and RNA-seq results. Genes associated with Wnt signaling tended to be downregulated in MZ*udu* mutant embryos, so Dr. Margot Williams in the LSK lab induced Wnt signaling in the MZ*udu* mutant embryos using lithium chloride (Ross and Bonner, 2012), but this also failed to restore mesoderm development or axis extension in the embryos (Williams, unpublished).

Ultimately, it may be difficult to identify a single pathway that can suppress the mesoderm patterning and cardiac defects in the MZ*udu* mutant embryos. The misregulation of

several morphogens including *fgf*, *bmp*, *wnt*, and RA signaling at early gastrulation stages make it likely that deviations in many pathways contribute the phenotypes. The shear number of misregulated pathways would make it technically difficult to test their ability to ameliorate the phenotypes in MZ*udu*$^{-/-}$ embryos, especially if multiple pathways must be tested simultaneously.

4.3 *udu* and *nkx2.5*

Many transcription factors are differentially expressed in MZ*udu* and Z*udu* mutant embryos. In particular, we were interested in the relationship between Gon4l and the cardiac transcription factor *nkx2.5*. Gon4l is secondarily linked to *Nkx2.5* through the transcription factor Yy1, which has been shown in mouse to regulate the expression of *Nkx2.5* (Beketaev, 2015; Gregoire et al., 2013). In Chapters 2 and 3, we show that *nkx2.5* expression is reduced in Z*udu*$^{-/-}$ embryos at later stages of development and almost absent in MZ*udu*$^{-/-}$ embryos; we also provide evidence that Gon4l interacts with the regulatory regions of *nkx2.5*. With genetic interaction studies, we demonstrate that *nkx2.5* and *udu* interact in the maintenance of ventricular identity. Furthermore, DamID-seq analysis found that Gon4l is associated with the regulatory regions of *nkx2.5* (Williams et al., 2018). Our evidence strongly links Gon4l to the regulation of *nkx2.5* during ALPM patterning and cardiac chamber emergence. Despite this, overexpressing *nkx2.5* through heatshock at 22-somite stage is unable to restore ventricular identity in Z*udu* mutant embryos. Likewise, injecting *nkx2.5* into one-cell stage MZ*udu* mutant embryos was not sufficient to restore heart development (Budine, unpublished). In total, these findings argue that despite Gon4l regulating *nkx2.5* expression, restoring *nkx2.5* expression is unable to suppress any of the overt cardiac phenotypes in either MZ*udu*$^{-/-}$ or Z*udu*$^{-/-}$ embryos. A critical piece of information needed to understand the relationship between Gon4l and *nkx2.5* is deciphering

when during development Gon4l regulates *nkx2.5*. DamID-seq data collected at tailbud stage indicates Gon4l may directly regulate *nkx2.5*. However, tb stage is before *nkx2.5* is expressed, which raises the question of why is Gon4l associating at the *nkx2.5* locus prior to transcription. Additionally, Nkx2.5 is repurposed during heart development from specifying CPCs to maintaining ventricular identity. It would be interesting to determine if Gon4l is regulates *nkx2.5* at these later stages and if the relationship is activating or repressing.

Data presented in this thesis and a recent publication on *udu* from our lab (Williams et al., 2018) argues that Gon4l regulates many transcription factors involved in mesoderm development. Exactly how Gon4l modulates the expression of these transcription factors is unknown. DamID-seq data show that Gon4l associates with the regulatory regions of thousands of genes (Williams et al., 2018). However, as a chromatin factor that lacks a DNA-binding domain (Liu et al., 2007b; Lu et al., 2010), Gon4l cannot be directly binding any of these genomic regions. We performed *de novo* motif analysis on the DamID-seq dataset and identified potential motifs of Gon4l association. These *de novo* Gon4l motifs were compared to known transcription factor motifs, and the closest matches identified. The known transcription factor motifs most similar to the motifs of Gon4l were enriched for functions in segment specification and heart development. Gon4l is known to complex with Yy1, HDAC1, and Sin3a (Lu et al., 2011); our motif results did identity Yy1 motifs as closely matched to Gon4l motifs, but many additional transcription factor motifs were found. These results open the intriguing question of if Gon4l has other binding partners. If so, could these binding partners provide the chromatin factor with mesoderm specific roles? It is possible that Gon4l complexes with transcription factors involved with cardiogenesis and hematopoiesis and that the partners binding localizes Gon4l

within the genome. An interesting follow-up to this discovery would be to use co-immunoprecipitation to test if Gon4l binds any of these transcription factors.

4.4 Zygotic functions of Gon4l

In Chapter 3, we present evidence that Gon4l has later zygotic functions in heart development that are separate and have a distinct mechanism from its maternal-zygotic functions. We demonstrate the Z*udu* mutant embryos have a reduction in the number of cardiomyocytes at 48 hpf that is a result of cell-cycle defects. The cell-cycle defect appears to affect the G1/S phase transition and could be a result of abnormal expression levels of *cyclin* genes. Furthermore, we show that unlike with the hematopoietic defects, the increase in *tp53* expression does not cause reduced number of cardiomyocytes in Z*udu* mutant embryos. This result suggests that the mechanisms underlying the cardiogenesis and hematopoiesis defects in Z*udu* mutant embryos are separate. A key outstanding question is if either activating expression of *ccne2* or inhibiting expression of *ccnd3* can rescue this cell-cycle defect.

Z*udu* mutant embryos also exhibit defects in the maintenance of ventricular identity. As discussed above, the defects in cardiac chamber patterning might arise through regulating the expression of *nkx2.5*. However, the inability of ectopic *nkx2.5* expression to suppress the defects in maintain ventricular identity argues for Gon4l regulating this process through additional pathways. Many transcription factors are involved in establishing and maintaining ventricular identity, notably *Hey2* and *Irx4* (Bao, 1999; Bruneau et al., 2001; Wu et al., 2013); full understanding regarding how Gon4l regulates this process will necessitate testing additional genes. The first step in identifying likely candidate genes would be performing RNA-seq on RNA isolated from hearts isolated from Z*udu* mutant embryos and identifying additional cardiac

patterning genes that are differentially expressed in the mutant hearts. Generating transgenic zebrafish lines driving expression of these genes of interest using a heat shock promoter would enable testing to see if these candidate genes can restore cardiac chamber patterning in Z*udu* mutant embryos.

4.5 Defining how Gon4l modifies the epigenetic landscape

It remains unclear Gon4l decides where to localize within the genome and how association with the protein regulates gene expression. The cooperation between Gon4l and Hdac1 (Lu et al., 2011) indicates that it is likely that Gon4l modifies the epigenetic landscape near genes to modify their expressions. It is important to note that this interaction of Gon4l with Hdac1 does not cause ubiquitous loss of acetylation. The phenotypes in MZ*udu*$^{-/-}$ embryos could not be suppressed through addition of trichtostatin A (TSA), an Hdac1 inhibitor (Budine, unpublished), implying that Gon4l has a level of specificity in regulating acetylation of the genome.

The key outstanding question in defining the role of Gon4l in development is learning how Gon4l association modifies the epigenetic landscape. Does Gon4l preferentially confer one type of chromatin modification? Does association with the chromatin factor make a gene more or less accessible? Assays of chromatin in Gon4l mutant embryos would enable us to define the epigenetic landscape changes that occur in *udu* mutant embryos. Two potential methods to address these questions are Chromatin Immunoprecipitation with sequencing (ChIP-seq) or Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), both of which present benefits and challenges (Buenrostro et al., 2015; Landt et al., 2012). Gon4l does not directly bind DNA, therefore, pull-down of Gon4l for ChIP-seq will likely be very difficult, if at

all possible. Another complication is there are no validated Gon4l antibodies in zebrafish, mouse, or human, let alone ChIP-grade antibodies. These issue make performing ChIP-seq by pulling-down Gon4l a difficult prospect. An alternate approach to ChiP-seq would be to use antibodies pull-down histone modifications rather than using antibodies for Gon4l. One downside is that Gon4l may affect many types of histone modifications, necessitating several rounds of ChIP-seq. ATAC-seq would overcome this difficulty by providing a whole-scale view of the chromatin landscape in MZ*udu* mutant embryos (Buenrostro et al., 2015). ATAC-seq would provide information about how distributions of hetero and euchromatin are altered in MZ*udu* mutant embryos, but it would not differentiate between the regions directly changed by Gon4l and downstream alterations. However, we could overlay our DamID-seq data with ATAC-seq to differentiate between the direct and indirect epigenetic modifications. Chromatin accessibility or association assays would enable us to gain a fuller understanding of the mechanisms of Gon4l in mesoderm development.

4.6 The roles of GON4L in human disease and development

GON4L is conserved in primates and humans, but little is known about it in humans. Intriguingly, *GON4L* has a primate specific partial duplication that results in a paralog, *YY1AP*, consisting mainly of the YY1-binding domain (Kuryshev et al., 2006). There have been a few studies implicating mutations in *GON4L* in human disease, especially cancer (Lezcano, 2018; Renieri et al., 2014). Essentially nothing is known about GON4L during early development. This may be a result of germline mutations in *GON4L* being incompatible with human life. Another possibility is that YY1AP can compensate for loss of GON4L in primates. Therefore, the absence of knowledge of how GON4L affects human development could be because there are no

overt defects. However, considering the myriad phenotypes of the *udu* zebrafish mutants, it seems more likely that mutations in *GON4L* result in spontaneous abortions during the first few weeks of human pregnancy.

To begin to address these questions, the LSK lab commissioned a knock-out of *GON4L* in H9 hESCs from the Genome Technology Access Center at Washington University in St. Louis. Three lines with bialleic *GON4L* disruptions were generated, each of them created frame shifts in the second exon that are hypothesized to result in early stops and non-sense mediated decay (Beltcheva, Kyaw Thu, Budine, unpublished). The $GON4L^{-/-}$ hESCs differentiate into cardiomyocytes when subjected to modulation of WNT/B-CATENIN signaling (Lian et al., 2012). The $GON4L^{-/-}$ hESCs had modest reductions in phosphorylated B-CATENIN, and it is postulated that the mutant cells are more sensitive the changes in WNT signaling (Beltcheva and Kyaw Thu). It is unknown what cell types the $GON4L^{-/-}$ hESCs differentiate into when not subjected to directed differentiation. Unbiased differentiation methods such as embryoid bodies could provide insight into this question (Itskovitz, 2000; Lin, 2014).

4.7 Conclusions

In summary, we have established novel roles for Gon4l in heart development from the earliest patterning of the nascent mesoderm to the establishment and maintenance of ventricular identity. We demonstrate that in maternal-zygotic loss of *udu* expression, mesoderm patterning is affected with an almost complete loss of ALPM mesoderm development. Furthermore, we show that in Z*udu* mutant embryos, heart development is proceeds normally until 24 hpf, then Gon4l is required for cardiomyocyte proliferation and maintenance of the ventricular fate. Outstanding questions remain, especially regarding how Gon4l changes the epigenetic landscape during

development. Additionally, it will be important to identify another other binding partners of Gon4l during mesodermal tissue development. Better understanding of how Gon4l regulates heart development will contribute to understanding of the regulatory networks that underpin cardiogenesis. Advances in unraveling the epigenetic control of this process could aid in improving methods of differentiating cardiomyocytes *in vivo* and in diagnosing CHDs.

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