Regulation of Primitive Erythropoiesis in *Xenopus laevis*

by

Mizuho Stacy Mimoto

A DISSERTATION

Presented to the Department of Cell & Developmental Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

August 2010

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Mizuho Stacy Mimoto

has been approved

Jan Christian, Ph.D., Dissertation Advisor

Ronen Schweitzer, Ph.D., Committee Chairman

James Lundblad, M.D., Ph.D., Committee Member

Alex Nechiporuk, Ph.D., Committee Member

Peter Rotwein, M.D., Committee Member

Marcel Wehrli, Ph.D., Committee Member

Acknowledgements

Thank you to everyone that has supported me and guided me along this path. First and foremost, thank you to my mentor Jan. I joined your lab because it was very important to me to learn to do science well and with integrity. I still firmly believe that it was an excellent choice in both these respects, and I aspire to live up to your example. Thank you to everyone in the Christian Lab past and present for your insightful conversations, (very) entertaining company and good taste in music.

Thank you to my committee (Ronen, Jim, Peter, Alex, Marcel and Jan) for keeping me on track.

Thank you to Lili Yamasaki and Kenro Kusumi. You believed in me as a much younger student and your enthusiasm for science shaped my view of the world. I continue to value your friendship and wisdom.

Thanks to my family, Pat, Satoru, and Ken and to all my grandparents for your unconditional love and support. And of course, thanks and love to my darling husband Jon. You inspire me daily with your creativity and love of science and make everything else worthwhile.

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List of Abbreviations

AGM	Aorta Gonad Mesonephros
AML	Amyeloid Leukemia
AP	Animal Pole
A/P	Anterior/Posterior
APC	Adenomatous Polyposis Coli
β-gal	β-galactosidase
BMP4	Bone Morphogenetic Protein 4
BSA	Bovine Serum Albumin
CamK	Calmodulin Kinase
Cdc42 GEF	Cdc42 Guanine Nucleotide Exchange Factor
CE	Convergent Extension
CKI	Casein Kinase I
CtBP	C-terminal Binding Protein
DKKI	Dikkopf-related Protein 1
Dll2	Distalless 2
DLP	Dorsolateral Plate
DM	Dorsal Mesoderm
DMZ	Dorsal Marginal Zone
Dsh	Disheveled
D/V	Dorsal/Ventral
E	Embryonic Day
ES	Embryonic Stem
FOG	Friend of GATA
Fz	Frizzled
GSK3β	Glycogen Synthase Kinase 3β
HEK293	Human Embryonic Kidney 293
HeLa	Henrietta Lacks
Hpf	Hours post-fertilization

HSC	Hematopoietic Stem Cell
Id2	Inhibitor of DNA binding 2
IHh	Indian Hedgehog
JNK	Jun NH_2-terminal Kinase
LEM	Leading Edge Mesoderm
LMO2	Lim-only Protein 2
LRP5/6	Low-density Lipoprotein Receptor-related Protein 5/6
MBS	Modified Barth's Saline
MBT	Mid-Blastula Transition
MO	Morpholino Oligo
Mta3	Metastasis-associated Protein 3
NAM	Normal Amphibian Medium
NCAM	Neural Cell Adhesion Molecule
NFAT	Nuclear Factor of Activated T cells
NuRD	Nucleosome Remodeling and Deacetylase
ODC	Ornithine Decarboxylase
PBS	Phosphate Buffered Saline
PCP	Planar Cell Polarity
PE	Posterior Epiblast
PKC	Protein Kinase C
qPCR/qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RBC	Red Blood Cell
SCA2	Stem Cell Antigen-related 2
SCL	Stem Cell Leukemia
St.	Stage
TCF/LEF	T-Cell Factor/Lymphoid Enhancer Factor
TLR4	Toll-like Receptor 4
TnC	Cardiac Troponin C
TRIL	Toll-like Receptor Interactor with Leucine-rich repeats
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

VBI	Ventral Blood Island
VE	Visceral Endoderm or Ventrolateral Ectoderm (Ch. 4)
VMZ	Ventral Marginal Zone
WBC	White Blood Cell
Wg	Wingless
Wnt	Wingless and int-related
XPOX2	*Xenopus* Myeloperoxidase 2

Abstract

Primitive erythropoiesis is the process that gives rise to the first red blood cells in the vertebrate embryo. It is regulated by signals dependent on GATA transcription factors that originate both within hematopoietic cells as well as in the surrounding microenvironment. In our model system, *Xenopus laevis,* GATA factors have distinct functions that are required to support primitive erythropoiesis in cells that are fated to form blood, as well as in cells that comprise their surrounding environment. In the first study, we have examined the cell-autonomous role of the GATA-transcriptional co-factor, Friend of GATA (FOG) during primitive erythropoiesis. Although FOG is known to be required for primitive erythropoiesis in the mouse, its role in *Xenopus* and the general mechanism(s) by which it functions during erythropoiesis are unclear. In the studies presented herein, we have established a requirement for FOG during *Xenopus* primitive erythropoiesis and demonstrated that depletion of endogenous FOG results in increased apoptosis of primitive erythrocytes. Using various mutant FOG constructs, we have performed structure-function analysis, to uncover a novel requirement for FOG interaction with the Nucleosome Remodeling and Deacetylase (NuRD) complex during primitive erythropoiesis.

In the second study, we have examined the requirement for GATA-2 in non-blood forming cells of the ectoderm during primitive erythropoiesis. Using microarray analysis we have found that the non-canonical and canonical Wnt pathways are reciprocally regulated downstream of ectodermal GATA-2. In addition, we have identified a novel gene xTRIL as positively regulated by GATA-

2. Initial studies indicate that xTRIL is also required for erythropoiesis and sufficient to activate non-canonical Wnt signaling. Our preliminary data support a hypothesis in which non-canonical Wnt signaling is activated downstream of GATA-2, possibly in an xTRIL-mediated fashion, and that these events are required to secondarily inhibit canonical Wnt signaling to allow blood progenitors to exit the cell cycle and adopt a hematopoietic fate.

CHAPTER 1

Introduction

Insight into how blood develops is intrinsically valuable in furthering our understanding of basic body morphogenesis and organ patterning. In addition, development of adult cancers often involves the reactivation or misregulation of latent embryonic programs. For example, inappropriate GATA activity has been shown to be the underlying phenomenon in several forms of leukemia (reviewed in Shimizu et al., 2008), while dysregulation of the Wnt pathway is the source of many types of malignancy, both hematopoietic and non-hematopoietic in origin (Kokolus and Nemeth, 2010). A complete understanding of the functions of these proteins during development and how they are regulated in the normal, physiologic context will therefore aid in the diagnosis and development of therapies when they become dysregulated in disease.

Xenopus laevis as a model system for primitive erythropoiesis

We have chosen *Xenopus laevis* as the model system in which to study primitive erythropoiesis for a variety of reasons. Several of the most salient are that first, *Xenopus* is a vertebrate and as such shares a high degree of homology to higher vertebrates. Second, unlike in mammals, *Xenopus* embryogenesis takes place externally, permitting us to access these embryos for manipulation and observation. They are also uniquely pigmented such that the dorsal and ventral aspects may be easily distinguished on inspection beginning at the four-

cell stage. These attributes are particularly useful for the types of studies to be described in the following chapters. For example, we are able to examine the effects of overexpressing or knocking down genes in a temporally and spatially controlled fashion by targeting injection of mRNAs or anti-sense morpholino oligos to the ventral side of the embryo, where prospective blood forming cells reside during early development. We are also able to examine the relationship between various tissue layers using established explant and culture systems to disrupt or facilitate their interaction. These embryos may then be returned to culture where we can monitor the effect of these manipulations across subsequent developmental stages. Further, my main interest is in studying primitive erythropoiesis, a process that takes place very early during development in structures that span only a few microns in the mouse embryo. By comparison, *Xenopus* embryos are remarkably large, measuring approximately one millimeter in diameter at the one-cell stage. Finally, *Xenopus* embryos are able to acquire oxygen via direct exchange through the epidermis, which prolongs their survival in the absence of red blood cells (RBCs), thus permitting us to study defects in erythropoiesis for extended periods of time. Together, these attributes make *Xenopus* an excellent system in which to approach the questions we have set out to address.

Overview of Vertebrate Hematopoiesis

In vertebrates, there are two waves of blood development, termed primitive and definitive hematopoiesis (Fig.1-1). Primitive hematopoiesis is a

transient process that primarily gives rise to nucleated RBCs. It was thus initially termed “primitive” in mammals because these early erythrocytes resemble those found in more primitive vertebrates, such as birds, frogs and fish, which retain their nuclei throughout adult life. This terminology continues to be used across vertebrate species to distinguish primary and secondary waves of hematopoiesis, which are spatially and temporally distinct and also differ in the mechanisms by

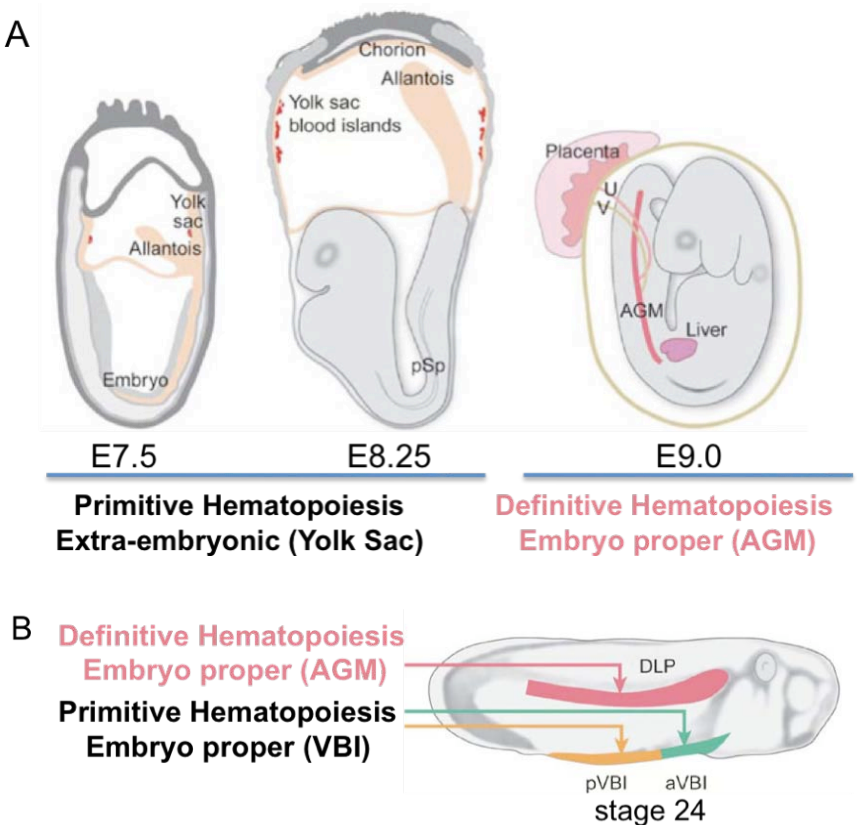

Figure 1-1. Sites of primitive versus definitive hematopoiesis in mammalian and amphibian embryos. (A) In mammals, such as the mouse depicted above, primitive hematopoiesis takes place extra-embryonically in the yolk sac blood islands (in red), whereas definitive hematopoiesis initiates intra-embryonically the aorta-gonad-mesonephros (AGM) region. (B) In *Xenopus*, primitive hematopoiesis takes place intra-embryonically in the ventral blood island (VBI). Definitive hematopoietic precursors are derived from a distinct population of cells that originate in the dorsolateral plate (DLP) mesoderm. Definitive hematopoiesis does not initiate until much later and does not contribute to the primitive circulation (Kau and Turpen, 1983). *Adapted from (Dzierzak and Speck, 2008).*

which they produce their respective lineages. During primitive hematopoiesis, mesodermally derived lineage-specific blood progenitors differentiate into mature hematopoietic cells. By comparison, during definitive hematopoiesis, a single multi-potent hematopoietic stem cell (HSC) has the ability to give rise to cells in all of the different blood lineages (reviewed in (Dzierzak and Speck, 2008).

Mammalian primitive hematopoiesis takes place extra-embryonically in the yolk sac blood islands (Fig. 1-1A). This begins at about embryonic day (E)7.0 of murine development (two weeks of human development) and terminates at E9.0 (four to five weeks in humans) (Palis et al., 1999). At this time, definitive hematopoiesis initiates intra-embryonically in the area encompassing the dorsal aorta, genital ridge, and developing kidney, termed the aorta-gonad-mesonephros (AGM; Fig. 1-1A) (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). The site of definitive hematopoiesis then migrates to the fetal liver and spleen before coming to reside in the bone marrow where in humans it persists throughout adult life. Transitions between these sites occur by seeding of each subsequent hematopoietic organ with HSCs, which are transported via the fetal circulation (Bertrand et al., 2006; Yokota et al., 2006).

Anatomy and timeline of primitive erythropoiesis in *Xenopus laevis*

The analogous structure in *Xenopus laevis* to the mammalian yolk sac blood islands is an intraembryonic structure in the tailbud stage embryo [24-48 hours post fertilization (hpf)] called the ventral blood island (VBI) (Fig. 1-1B). The origins of blood development in *X. laevis*, however, begin much earlier. Extensive

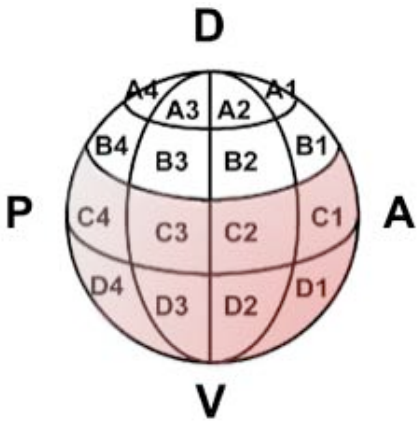

Figure 1-2. *Xenopus laevis* embryo at the 32-cell stage with blastomeres marked according to standard nomenclature. Lineage tracing studies indicate that primitive erythrocytes are derived, at least partially, from all C- and D-tier blastomeres (shaded) (Lane and Sheets, 2002).

lineage tracing studies at the 32- and 64-cell stage (3 hpf) have demonstrated that red blood cells derive from all C- and D-tier blastomeres (equatorial and vegetal blastomeres; shaded in Fig. 1-2) (Lane and Sheets, 2002; Lane and Smith, 1999).

During gastrulation, cells of the ventral mesoderm receive signals from the overlying ectoderm that are required in order for them to be specified to an erythroid fate (Dalgin et al., 2007; Maeno et al., 1996; Maeno et al., 1994b). From a developmental perspective, the terms commitment, which includes specification and determination, and differentiation have precise and distinct implications that may be useful to define. During commitment, cells are specified to a particular fate, such as the erythroid lineage, and this is reflected in the expression of markers such as *Stem cell leukemia (SCL), LIM-only protein 2 (LMO-2), AML (Amyeloid Leukemia)*, *GATA-2*, and *GATA-1* (Kelley et al., 1994; Mead et al., 2001; Mead et al., 1998; Tracey et al., 1998; Wadman et al., 1997; Zon et al., 1991). These genes are first detected in a subset of mesodermal cells following gastrulation (approximately 16 hpf). Cells are said to be specified when they are capable of differentiating autonomously when placed in a neutral environment, as is the case with the ventral mesoderm once the window during

which ectodermal signals are required for hematopoiesis has passed (discussed in detail below). Cells may lose this specification or be induced to take on a different fate if they are transplanted to another region of the embryo, where inhibitory signals can potentially reverse commitment. Once cells have become determined with a given fate, they will adopt that fate regardless of external signals. Finally, during differentiation, cells take on specialized structures or functions that are relevant to the particular tissue type or organ in which they

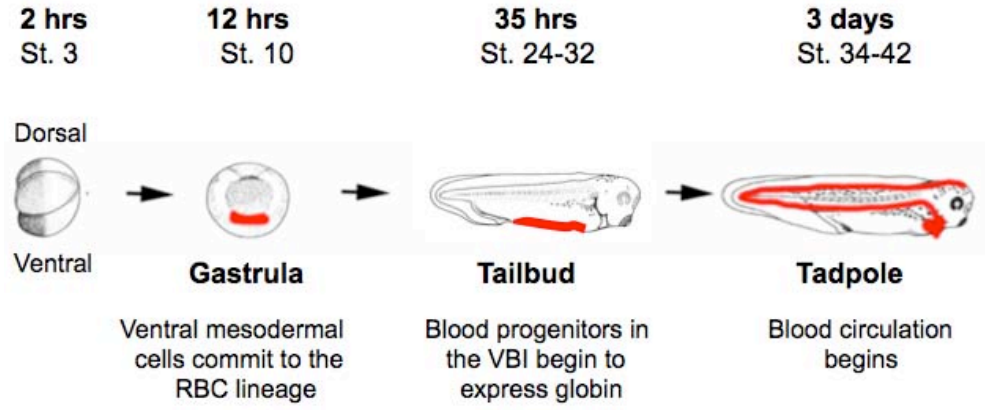

Figure 1-3. Overview of primitive erythropoiesis during *Xenopus* development. During *Xenopus* development, cells of the ventral mesoderm (red bar) first become specified to an erythroid fate during gastrulation. By the tailbud stage primitive erythrocytes are undergoing differentiation and express the marker *globin*. The heart also initiates contraction at this stage. By the tadpole stage, primitive erythrocytes may be visualized in circulation and embryos can be bled for analysis of red blood cell morphology and number. *Adapted from (Nieuwkoop and Faber, 1994).*

reside. Thus, between stages (st.) 24-32 (26-42 hpf), RBCs begin to differentiate and to express *globin* genes, essential components of the hemoglobin complex required to bind and transport oxygen. *Globin* expression begins in the anterior VBI just caudal to the liver anlage at about stage 24 (26 hpf) and extends posteriorly as more hemoglobin is synthesized. At roughly stage 35-6 (50 hpf), the heart begins to beat and RBCs begin to circulate in the embryo (Zon, 1995).

At the tadpole stage (st. 42; 80 hpf) all primitive RBCs have entered the circulation. See Figure 1-3 for an overview and timeline of primitive erythropoiesis in *Xenopus laevis*.

GATA-1 and GATA-2 are essential for primitive erythropoiesis

Primitive hematopoiesis is regulated by both intrinsic, or cell autonomous signals and extrinsic, or cell non-autonomous signals. Several members of the GATA family of transcription factors play an essential, cell autonomous role in directing erythroid development. There are six vertebrate GATAs of which GATA-1-3 are involved in hematopoiesis and GATA-4-6 are involved in development of other organs such as the heart, lung, GI tract and nervous system (Patient and McGhee, 2002)]. Only GATA-1 and GATA-2 play essential roles in erythropoiesis (Fujiwara et al., 1996; Tsai et al., 1994). The requirement for GATA protein function during hematopoiesis is evolutionarily well conserved from fish to humans (Dalgin et al., 2007; Fujiwara et al., 1996; Fujiwara et al., 2004; Galloway et al., 2005; Lyons et al., 2002; Nichols et al., 2000; Pevny et al., 1991; Shimizu et al., 2008; Tsai et al., 1994). Mice deficient in either GATA-1 or GATA-2 die *in utero* from failure of primitive erythropoiesis, though the defect varies somewhat based on the individual functions of each protein. Although GATA-2 null mice still produce mature RBCs, they are drastically reduced in number (Tsai et al., 1994). In contrast, erythrocytes in GATA-1 null mice, fail to properly mature (Pevny et al., 1991), which demonstrates that GATA-1 plays a distinct and complementary role in RBC differentiation. Loss of both GATA-1 and GATA-2

abrogates all primitive erythropoiesis, thus the milder phenotypes in the individual mutants suggest that they can compensate for one another to some degree. These studies and others indicate that in addition to its role in non-hematopoietic cells, GATA-2 is also required within hematopoietic mesoderm for RBC progenitor maintenance.

In *Xenopus*, maternally expressed GATA-2 is present at low levels in the early embryo. At roughly eight hpf, zygotic gene expression initiates and GATA-2 expression is first robustly detected by *in situ* hybridization in the animal pole at this time, consistent with its ectodermal function during gastrulation. Expression of GATA-2 subsequently expands into the ventral mesoderm of the embryo and specifically localizes to erythroid progenitors at stage 20 but declines by stage 26 (Kelley et al., 1994), consistent with its role in RBC progenitor maintenance (Tsai et al., 1994). Expression of GATA-1 is initiated during gastrulation (Zon et al., 1991) but is not detectable by *in situ* hybridization until stage 25, when transcripts localize to the VBI (Kelley et al., 1994). This pattern of expression supports studies indicating that GATA-1 represses expression of proliferative genes required to maintain a progenitor state (such as *GATA-2*; (Welch et al., 2004) and also directly activates genes required for differentiation to a RBC fate (such as *globin*; (Letting et al., 2004).

Friend of GATA (FOG) is an essential transcriptional cofactor required during primitive hematopoiesis

Friend of GATA (FOG) is a GATA transcriptional co-factor that is essential for many different GATA regulated processes, including hematopoiesis. Loss of FOG in the mouse causes a block in RBC differentiation, phenocopying the defect seen in *GATA-1* null animals (Tsang et al., 1998). FOG serves as both a transcriptional co-activator and co-repressor of GATA target genes (Cantor and Orkin, 2005; Letting et al., 2004; Pal et al., 2004; Svensson et al., 2000) but the mechanisms by which it does so are unclear. FOG has two known co-repressor-binding domains which mediate interactions with the Nucleosome Remodeling and Deacetylase complex (NuRD) (Hong et al., 2005; Lin et al., 2004) and the C-terminal Binding Protein (CtBP) (Deconinck et al., 2000; Katz et al., 2002). Both domains are highly conserved across evolution in a majority of FOG isoforms (Katz et al., 2002; Lin et al., 2004; Svensson et al., 2000), though only interaction with NuRD has been shown to have a function *in vivo* thus far (Fox et al., 1999; Katz et al., 2002; Walton et al., 2006).

Although the fact that FOG plays an essential role in hematopoiesis is well documented from flies to fish to mammals (Amigo et al., 2009; Fossett et al., 2001; Nichols et al., 2000; Tsang et al., 1998), its precise function during *Xenopus* primitive erythropoiesis has been the subject of debate due to conflicting data from overexpression studies in the frog (Deconinck et al., 2000). In Chapter Two, we have resolved these inconsistencies using loss of function analysis as a more stringent assay for FOG function. We have also taken advantage of this system to perform structure/function analysis by rescuing loss

of FOG with mutants deficient in FOG's known binding domains, in order to determine the function of these domains during primitive erythropoiesis.

Non-cell autonomous signals are required for primitive erythropoiesis

In addition to essential cell autonomous signals mediated by proteins such as FOG, many studies have shown that in *Xenopus* embryos, mesoderm must also receive external signals from the ectoderm during gastrulation in order to differentiate into RBCs (Dalgin et al., 2007; Maeno et al., 1996; Maeno et al., 1994b). During gastrulation (9 hpf), mesodermal cells begin to invaginate into the interior of the embryo on the vegetal aspect in a circumfrential fashion (Keller, 1991). Invagination begins on the dorso-anterior side (Fig. 1-4B) and is followed about two hours later by cells on the ventro-posterior side, forming a ring of

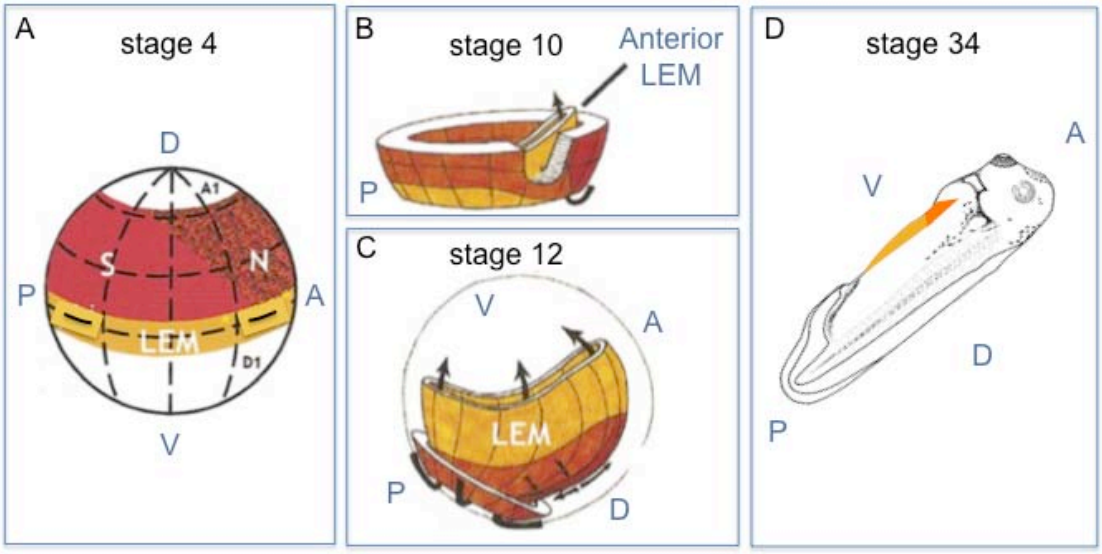

Figure 1-4. *Xenopus* gastrulation movements. Mesoderm that originates in the dorsal/anterior (D/A) or ventral/posterior (V/P) marginal zones of the embryo (A) invaginates and migrates through the embryo to the ventral aspect during gastrulation (B,C) where they contribute to the ventral blood island (orange = dorsoanterior contribution, yellow = ventroposterior contribution) (D). LEM = Leading Edge Mesoderm, S = Somites, N = Neural. See text for details. *Adapted from (Lane and Sheets, 2000).*

inwardly migrating cells (Fig. 1-4C). Both groups of cells, together termed the Leading Edge Mesoderm (LEM), will eventually emerge on the ventral side of the embryo [Fig. 1-4D; (Keller, 1991; Lane and Sheets, 2000)]. Although the dorso-anterior population will give rise to a small portion of the anterior VBI, the VBI will be predominantly derived from cells of the ventro-posterior aspect (Fig. 1-4D) (Lane and Sheets, 2002). As such, we target our manipulations (to be described later) in the early embryo to “ventral” or “ventroposterior” cells in order to affect regions of the embryo that will give rise to the majority of the red blood cell precursors.

During transit through the embryo, the mesoderm comes into close contact with the overlying ectoderm (Fig. 1-5A), which provides signals essential for blood formation. Although a number of different structures are formed from

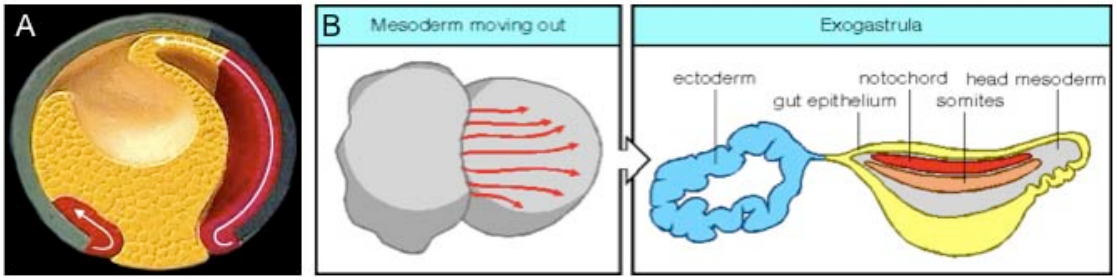

Figure 1-5. Gastrulation vs. Exogastrulation in *Xenopus* embryos. (A) Saggital view of gastrula stage embryo. Involuting mesoderm is depicted in red and overlying ectoderm is in green **(***Kozlowski and Tyler).* (B) Culturing embryos in a high salt solution induces exogastrulation. This prevents contact between ectoderm and mesoderm by causing them to migrate in opposite directions during gastrulation. Loss of contact between the ectoderm and mesoderm is sufficient to inhibit primitive erythropoiesis but not development of other mesodermally derived tissues. (*Gilbert, et al.*)

the mesoderm, the requirement for ectodermal signals is unique to hematopoietic cells. Culturing embryos in a high salt solution induces them to undergo a process called exogastrulation, such that the ectoderm and mesoderm migrate in

opposite directions and do not come into contact with one another (Fig. 1-5; Kikkawa et al., 2001). Other mesodermal derivatives, such as somites, head mesoderm and notochord, continue to differentiate in exogastrulae (Kikkawa et al., 2001; Ruiz i Altaba, 1992) however, expression of RBC differentiation markers is absent (Kikkawa et al., 2001). Data presented in Chapter Three indicate that specification may also be affected by loss of ectodermal contact. These studies demonstrate that within the developing mesoderm of the frog, hematopoietic cells are unique in their requirement for an external ectodermal signal for proper specification.

Interestingly, this requirement for non-cell autonomous signals is also conserved in mammals and birds (Belaoussoff et al., 1998; Miura and Wilt, 1969). In mouse, mesoderm that will eventually give rise to primitive erythrocytes develops from cells of the posterior epiblast (PE) as they migrate through the primitive streak. During transit through the primitive streak, the PE comes into close contact with cells of the overlying visceral endoderm (VE). When the PE and VE are dissociated by treatment with proteases at the pre- or early-streak stage (E6.0-E6.25) the PE fails to express *globin* (Belaoussoff et al., 1998). However, when co-cultured with VE, *globin* expression in the PE is restored, demonstrating that mouse VE has an inductive function similar to that of ectoderm in frogs. Moreover, the non-blood forming anterior epiblast also expresses *globin* when co-cultured with VE, indicating that the VE is sufficient to reprogram other cell types to a hematopoietic fate (Belaoussoff et al., 1998). These studies demonstrate that the need for extrinsic signals from adjacent germ

layers during development of primitive erythrocytes is conserved among vertebrates. The nature of this inductive signal is an intriguing question that will be addressed in Chapters Three and Four.

Ectodermal GATA-2 is both necessary and sufficient to induce erythropoiesis in the mesoderm

Early work by Mitsugu Maeno used explant and culture assays in *Xenopus* to confirm that ectodermal signals are required to enable the ventral mesoderm to form blood (Maeno et al., 1994b). Specifically, when ventral mesoderm, which is fated to become blood, is dissected at the gastrula stage (st. 10, 8 hpf) and cultured independently to the tailbud stage, it does not express *globin* (Maeno et al., 1994b) (Fig. 1-6A). However, when co-cultured with a segment of the overlying ectoderm, an equivalent segment of ventral mesoderm expresses *globin* at levels comparable to that of sibling whole embryos at the tailbud stage (Fig. 1-6B). This observation indicates that the ectoderm provides signals to the mesoderm that are required for blood formation. Subsequent studies established specific windows of development during which 1) the ectoderm is competent to provide inductive signals to the mesoderm and 2) the ventral mesoderm requires inductive signals from the ectoderm. Specifically, when taken prior to the mid-blastula transition (MBT; 8 hpf), which marks the onset of zygotic transcription, ectodermal explants are not yet able to support blood formation in the mesoderm (Maeno et al., 1994b). In addition, by the neurula stage (st. 16; 18 hpf) ventral mesoderm explants are competent to form blood independently (Maeno et al.,

1994b) indicating that hematopoietic fate has been specified by this stage. Together, these studies demonstrate that the ectodermal inductive signal required for hematopoiesis is generated and transmitted between stages 10 and 16. Work to be described in Chapter Three has further narrowed this window of development to facilitate identification of this signal.

Additional work by Maeno's group identified the transcription factor GATA-2 as being sufficient to render early (pre-MBT) ectoderm competent to form

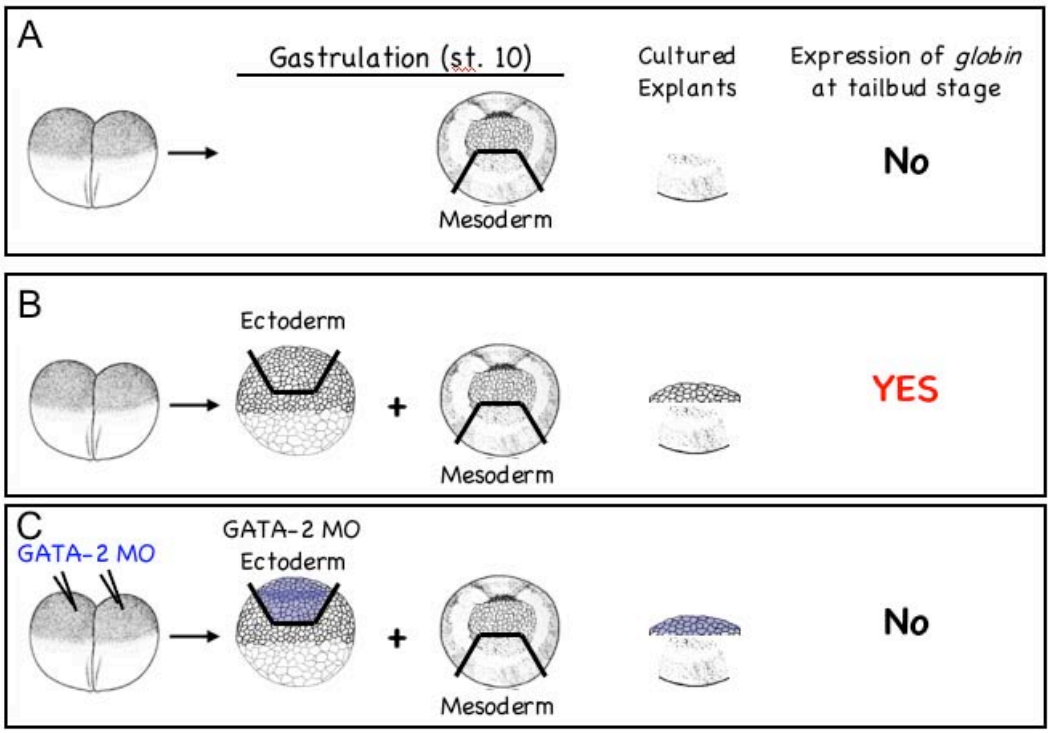

Figure 1-6. Schematic of ectoderm-mesoderm recombinant assays used to establish a non-cell autonomous requirement for ectodermal signals during primitive erythropoiesis. (A) Mesodermal explants dissected at the gastrula stage cannot support hematopoiesis when cultured alone to the tailbud stage (Maeno et al., 1994b). (B) Co-culture with explanted ectoderm from the same stage embryos rescues RBC formation in mesodermal explants (Chien et al., 2009; Maeno et al., 1994b). (C) Depletion of GATA-2 by morpholino oligo (MO) injection renders ectoderm unable to support primitive erythropoiesis when recombined with wild type mesoderm (Dalgin et al., 2007). *Images adapted from* (Nieuwkoop and Faber, 1994).

blood. Specifically, co-culture of stage 7 ectoderm injected with very low levels of

RNA encoding GATA-2 (8pg) could induce expression of *globin* in VM explants at levels comparable to those induced by co-culture with stage 10 ectoderm (Maeno et al., 1996). Loss-of-function studies from our lab subsequently showed that depletion of GATA-2 in the ectoderm renders it unable to support *globin* expression when recombined with wild type mesoderm (Dalgin et al., 2007; Fig. 1-6C). The studies described in Chapters Three and Four are aimed at identifying the nature of the ectodermal inductive signal(s) mediated by GATA-2.

Because GATA-2 is transcription factor, it is likely activating expression of a secondary signal that is either secreted or membrane-bound, and would thus be in a position to signal between germ layers. Several molecules have been proposed as candidates for this soluble signal including Bone Morphogenetic Protein 4 (BMP4) in frogs and Indian Hedgehog (IHh) in mice. Both proteins are secreted signaling molecules and are expressed during the appropriate developmental stages to mediate a signal required for hematopoiesis (Dyer et al., 2001; Maeno et al., 1996). However, several lines of evidence argue against either BMP4 or IHh as being the sole mediator of erythropoietic inducing signals. First, in *Xenopus*, constitutive activation of BMP4 signaling cannot rescue RBC formation from gastrula-stage mesodermal explants, indicating that expression of BMP4 alone is not sufficient to induce erythropoiesis (Dalgin et al., 2007). Second, in the mouse, although IHh is expressed in visceral endoderm and is sufficient to induce blood formation from isolated posterior epiblast, mice deficient for IHh do not display defects in hematopoiesis (St-Jacques et al., 1999). As described in Chapters Three and Four, we have used microarray

analysis as an unbiased approach to address the question of which signaling pathways downstream of GATA-2 are required for primitive erythropoiesis.

Several exciting avenues of exploration were identified in this screen. The first is that non-canonical and canonical Wnt signaling pathways appear to be reciprocally regulated downstream of GATA-2. Second, we have identified a novel protein, xTRIL, which appears to be both required for erythropoiesis and sufficient to activate non-canonical Wnt signaling. The following sections are intended to provide an overview of the relevant Wnt signaling pathways and to give a brief introduction to xTRIL.

Overview and discovery of the Wnt family of proteins

Wnt (for wingless and int1-related) proteins are a family of secreted signaling molecules that are important for many different biological processes during development including cell fate specification, proliferation, and tissue morphogenesis (reviewed in (Chien et al., 2009; MacDonald et al., 2009). They have also been implicated in tumor suppression during adult life (Liang et al., 2003). Disruption of the Wnt pathway is associated with developmental defects and adult malignancy in multiple contexts, including during hematopoiesis (discussed below and in (Kokolus and Nemeth, 2010). The *wingless* (*wg*) gene was initially identified in *Drosophila* by two groups as a mutation that produces defects in wing formation (Nusslein-Volhard and Wieschaus, 1980; Sharma and Chopra, 1976). The first Wnt gene in mammals was identified as a locus of preferential *int*egration of the mouse mammary tumor virus (*int-1*) that produced

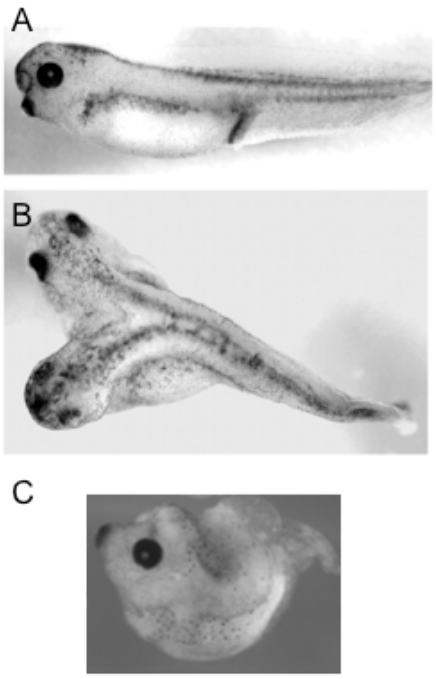

Figure 1-7. Duplication of the axis in *Xenopus* embryos. (A) Normal embryo at the late tailbud stage. (B) Tailbud stage embryo in which the axis has been duplicated due to hyperactivation of canonical Wnt signaling on the ventral side. *Wallingford and Habas (2005)* (C) Tailbud stage embryo exhibiting spina bifida due to disruption of non-canonical Wnt signaling required for convergent extension movements (*Jan Christian*).

tumors (Nusse and Varmus, 1982). These two independent discoveries were unified when Nusse and colleagues cloned the *Drosophila int-1* gene and mapped it to the *wg* locus (Rijsewijk et al., 1987). Subsequent identification of additional Wnt family members and Wnt proteins in other species were made by homology mapping. Currently, 19 Wnt ligands have been identified in mice and humans (Chien et al., 2009). Within the Wnt family, there are two major subdivisions that are distinguished by whether they signal via stabilization of β-catenin (the *canonical* or *β-catenin mediated Wnt pathway*) or are β-catenin independent (the *non-canonical* or *non-β-catenin mediated Wnt pathway*).

Historical basis for the distinction between the canonical and non-canonical Wnt signaling pathways

The historical distinction between the canonical and non-canonical Wnt pathways is based on the observation that some Wnt ligands, when overexpressed on the ventral side of a cleavage-stage *Xenopus* embryo, can

induce a second dorsal axis, giving rise to an embryo with two heads and two sets of neural structures (Fig. 1-7B). These Wnts (Wnt-1, -3, -8, and -8b) (McMahon and Moon, 1989) (Christian et al., 1991b; Du et al., 1995) also promote cell adhesion (Torres et al., 1996) and induce transformation of C57mg mammary epithelial cells (Wong et al., 1994). These functions were shown to be mediated by stabilization of β-catenin and were described as “canonical.” A second class of Wnt ligands (Wnt-4, -5a, and -11) when overexpressed in the same fashion in *Xenopus* embryos did not produce a duplicated axis, but instead caused defects in gastrulation and in convergent extension (CE; Fig. 1-7C), the movements required for vertebrate axis elongation (Du et al., 1995; Moon et al., 1993). Unlike canonical Wnts, these Wnts reduced cell adhesion, did not transform C57mg cells and did not rely on β-catenin for their effects (reviewed in (Chien et al., 2009). These Wnt ligands were thus referred to as mediating a “non-canonical” Wnt signal. Based on these early observations and many subsequent studies, canonical Wnt signaling is typically associated with inducing cell proliferation (as with the mouse mammary cells) and determining cell fate (as with the induction of the second axis) in many different contexts (Chien et al., 2009). By comparison, non-canonical Wnt signaling regulates cytoskeletal rearrangements required for tissue polarity and morphogenetic movements during development (Wallingford et al., 2002a), and promotes exit from pluripotency by promoting specification and differentiation of stem and progenitor cells (Nusse et al., 2008; Vijayaragavan et al., 2009; Zhang et al., 2008).

Canonical Wnt signaling is required to establish both dorsal and ventral fates during distinct phases of development

One of the primary functions of the Wnt pathway during *Xenopus* embryogenesis is establishment of the dorsal/ventral (D/V) axis. Beginning at fertilization, maternally-derived canonical Wnt signals are required for *dorsal* axis specification (Cha et al., 2008; Heasman et al., 1994; Tao et al., 2005). Somewhat later, following initiation of zygotic transcription at the mid-blastula transition (MBT; stage 8), zygotically-derived canonical Wnt signals are required for *ventral* mesoderm specification. Thus the role of canonical Wnts switches from promoting dorsal fate to promoting ventral fate midway through early development.

Wnt5a, Wnt8 and Wnt11 are the primary Wnt ligands expressed in the embryo prior to the end of gastrulation (Christian et al., 1991a), and each is important for different aspects of D/V axis specification. While maternal Wnt5a is symmetrically distributed within the vegetal cytoplasm at the one-cell stage (Moon et al., 1993), expression of Wnt11 is concentrated on the dorsal side as a result of cortical rotation of the vegetal cytoplasm, which occurs upon sperm entry (Schroeder et al., 1999). Recent studies have demonstrated that during early cleavage stages maternally expressed Wnt5a and Wnt11 are required for initial specification of the dorsal axis in *Xenopus* embryos (Cha et al., 2008; Tao et al., 2005). Interestingly, unlike subsequent developmental stages during which these ligands act via the non-canonical Wnt pathway, Wnt5a and Wnt11 signal through β-catenin at this time. Maternal depletion of either ligand causes a loss of

dorsal and anterior structures (Cha et al., 2008; Tao et al., 2005), defects that are similar to those observed with maternal depletion of β-catenin (Heasman et al., 1994). Moreover, the defects caused by Wnt5a or Wnt11 depletion are rescued by co-expression of β-catenin (Cha et al., 2008; Tao et al., 2005), indicating that these ligands are individually required to specify dorsal fate and that they do so by a β-catenin-dependent mechanism.

As described above, ectopic ventral expression of either β-catenin or of canonical Wnts (such as Wnt1 and Wnt8, but not Wnt11 or Wnt5a) during early cleavage stages dorsalizes the embryo and results in a duplicated axis, suggesting that the classical canonical Wnt ligands promote dorsal fate. However, seminal work by Christian and Moon demonstrated that this hyper-dorsalized phenotype in response to Wnt1 or Wnt8 reflects an artifact of overexpression (Christian and Moon, 1993), as endogenous expression of classical canonical Wnts does not initiate until the early blastula stage (st. 8-9), when Wnt8 begins to be expressed in the ventral marginal zone (Christian et al., 1991b). Consistent with this idea, dorsal injection into cleavage-stage embryos of Wnt8 cDNA, which is not transcribed until MBT and thus more closely reflects the timing of endogenous Wnt8 expression, causes failure of dorsal structures to form (Christian and Moon, 1993). Thus, following MBT, canonical Wnt function switches from promoting dorsal fate to promoting ventral fate, indicating that the context in which ligands are expressed is important in determining their ultimate developmental effects. Assays employing overexpression of Wnts and their downstream signaling components must therefore be interpreted with caution

and validated in the whole animal based on endogenous patterns and levels of expression.

Signaling mechanisms in canonical versus non-canonical Wnt pathways

The current model of canonical Wnt signaling (reviewed in (MacDonald et al., 2009) dictates that in the absence of Wnt ligand binding, β-catenin is constitutively phosphorylated and degraded by a complex including Axin, APC, CKI and GKS3β. Pathway activation occurs when Wnt ligands bind to the seven-

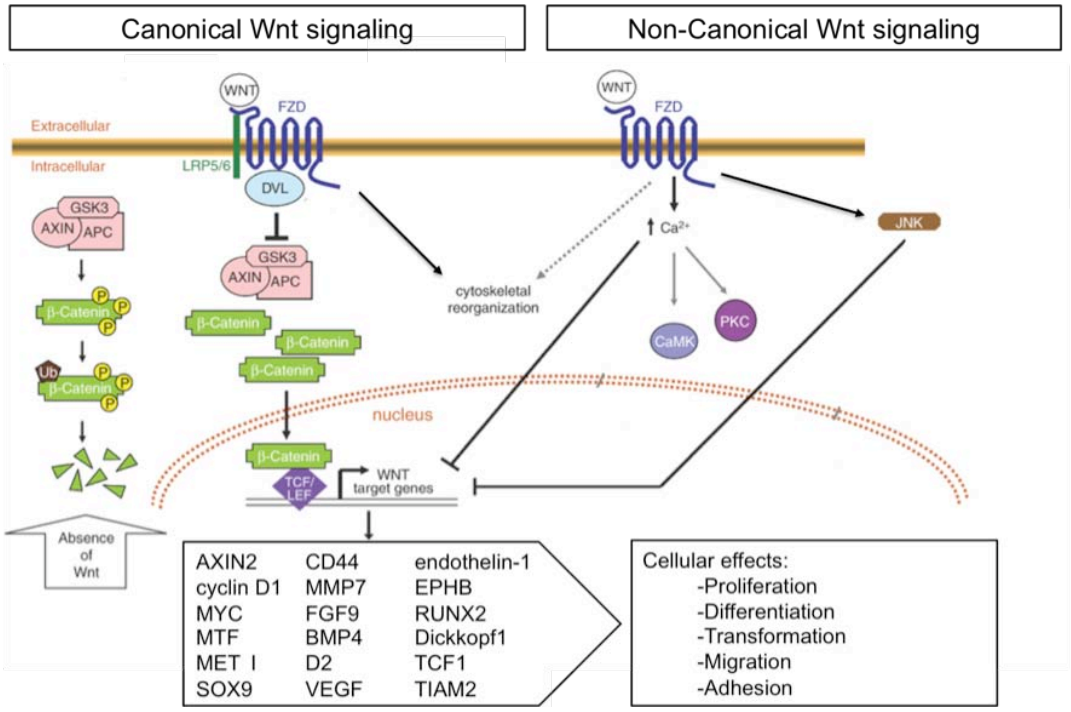

Figure 1-8. Schematic of canonical (β-catenin-mediated) and non-canonical (β-catenin independent) Wnt signaling pathways. In canonical Wnt signaling (depicted on the left), ligand binding induces stabilization of β-catenin, which then enters the nucleus to induce TCF/LEF mediated gene transcription**.** In non-canonical Wnt signaling (depicted on the right), ligand binding induces a number of downstream signaling molecules. Ca^{2+}/CamK, PKC and JNK are depicted here. Activation of these pathways has various cellular effects, including repression of canonical Wnt signaling. See text for more detail. *Adapted from (Chien et al., 2009).*

pass transmembrane receptor Frizzled (Fz) and its co-receptor low density lipoprotein receptor-related protein five or six (LRP5/6). Ligand binding causes recruitment and activation of the downstream effector protein Disheveled (Dsh). Recruitment of Dsh induces secondary recruitment of other proteins, including members of the β-catenin destruction complex, resulting in inhibition of the destruction complex and stabilization of β-catenin. β-catenin accumulates in the cytoplasm and enters the nucleus where it can participate in activation of downstream targets via binding to partner transcription factors such as T-Cell Factor/Lymphoid Enhancer Factor (TCF/LEF) (Chien et al., 2009; MacDonald et al., 2009) (Fig. 1-8). Several recent studies have demonstrated that canonical Wnt signaling promotes proliferation at the expense of differentiation during definitive hematopoiesis. Loss of canonical signaling either in mice deficient for Wnt3a or that overexpress the Wnt inhibitor DKK1 have reduced numbers of HSCs and fail to rescue lethally irradiated hosts in serial transplantation assays (Fleming et al., 2008; Luis et al., 2009). By contrast, conditional activation of β-catenin in the hematopoietic compartment leads to increased HSC proliferation. Interestingly, HSCs from these animals also fail in long-term repopulation assays, due to stem cell exhaustion from excessive proliferation (Kirstetter et al., 2006; Scheller et al., 2006). Together, these studies indicate that canonical Wnt signals play a key role in expansion and maintenance of HSCs during definitive hematopoiesis.

The non-canonical, or non-β-catenin mediated, Wnt pathway can be subdivided into a number of distinct divisions (Fig. 1-8) (reviewed in (Gao and

Chen, 2010; Slusarski and Pelegri, 2007). The two primary divisions that are the focus of studies proposed herein are the Wnt-Planar Cell Polarity (Wnt-PCP) pathway and the Wnt-Ca^{2+} pathway (Fig. 1-8). Similar to the canonical Wnt pathway, both the Wnt-PCP and Wnt-Ca^{2+} pathways are activated by Wnt binding to Fz, resulting in recruitment of Dsh to the cell membrane (Boutros et al., 1998; Slusarski et al., 1997b). At this point, the pathways diverge and downstream signaling events appear to require utilization of different domains of Dsh (Gao and Chen, 2010). The Wnt-PCP pathway signals via activation of Jun NH_2-terminal kinase (JNK) (Boutros et al., 1998; Yamanaka et al., 2002), and in some instances involves activation of small GTPase family members Rho, Rac and Cdc42 in a context-dependent fashion (Schlessinger et al., 2009). Signaling through the non-canonical Wnt pathway is essential for regulating cytoskeletal rearrangements during morphogenesis, and for tissue polarity, particularly during convergent extension. Disruption of this pathway either through over-activation or loss-of-function of PCP effectors results in abnormal or absence of cell polarity, respectively (Goto and Keller, 2002; Sokol, 1996; Wallingford et al., 2002a; Wallingford et al., 2000). Thus either gain-of-function or loss-of-function of non-canonical Wnt pathway components results in similar morphologic defects (Fig. 1-7C)

A second non-canonical Wnt pathway, the Wnt-Ca^{2+} pathway, results in subsequent binding and activation of a heterotrimeric G protein. G protein activation leads to an increase in intracellular Ca^{2+} (Slusarski et al., 1997a), which in turn can activate Protein Kinase C (PKC) and/or Cam Kinase II (CamKII)

(Kuhl et al., 2000; Sheldahl et al., 2003). Recent studies indicate that activation of the Wnt/Ca^{2+} pathway may be important for exit from a proliferative state and induction of hematopoietic mesoderm in a model using human embryonic stem (ES) cell differentiation (Vijayaragavan et al., 2009). In this system, the Wnt/Ca^{2+} pathway functions to dampen proliferative signals induced by the canonical Wnt pathway and thus promotes normal developmental progression from stem/progenitor cells to more differentiated cell types during definitive hematopoiesis (Vijayaragavan et al., 2009). Our findings described in Chapters Three and Four, provide evidence for a similar regulatory mechanism during primitive erythropoiesis, in which reciprocal regulation of canonical and non-canonical Wnt signals by GATA-2 is required to mediate developmental transitions between hematopoietic progenitors and differentiated primitive erythrocytes.

Thesis Outline

The remainder of Chapter One provides background for understanding primitive hematopoiesis and its regulation by extrinsic or non-cell autonomous signals from the ectodermal niche. It also covers events relevant to hematopoiesis during development in our model system, *Xenopus laevis*. Finally, I've provided a brief introduction to the Wnt signaling pathway and its functions during early *Xenopus* development, as this will be relevant to the studies discussed in Chapters Three and Four.

Chapter Two of this thesis discusses the role of a key hematopoietic transcriptional co-factor, *Friend of GATA* (*FOG*), in regulating primitive erythropoiesis from within hematopoietic cells. Using loss-of-function analysis, we have determined that FOG is required for primitive erythropoiesis in *Xenopus*. We have further determined that disruption of FOG results in an increase in the fraction of primitive erythrocytes undergoing apoptosis, indicating that FOG is required to support survival in these cells. Finally, we used FOG deletion mutants to perform a structure-function analysis in order to ascertain which known functional domains of FOG are relevant for primitive erythropoiesis. Our findings revealed a novel role for association of FOG with the Nucleosome Remodeling and Deacetylase (NuRD) complex during blood development. Together these studies establish that FOG is required to support primitive erythropoiesis in *Xenopus* and provide insight into the mechanism by which it does so.

Chapter Three describes the results and subsequent analysis of a microarray study that we initiated to identify genes downstream of GATA-2 in the ectoderm that are required for primitive erythropoiesis in the mesoderm. Using this approach, we found that the canonical and non-canonical Wnt pathways are reciprocally regulated downstream of GATA-2, and that this inverse regulation is required for normal primitive erythropoiesis.

Chapter Four describes characterization of the novel gene, xTRIL, identified by the microarray analysis presented in Chapter Three. Based on our preliminary studies, xTRIL appears to be required for primitive erythropoiesis and functions via activation of the non-canonical Wnt signaling pathway.

Finally, Chapter Five is a summary of the data presented in this thesis and a brief discussion of potential future experiments for which these studies have laid the groundwork.

CHAPTER 2

Friend of GATA (*FOG*) and FOG-NuRD interaction are required for primitive erythropoiesis in *Xenopus laevis*

Mizuho S. Mimoto and Jan L. Christian

Department of Cell and Developmental Biology

Oregon Health and Science University, School of Medicine

3181 SW Sam Jackson Park Road, L215

Portland, OR 97239-3098

PREFACE

This manuscript is currently in preparation.

- My contributions to this study include performing all experiments except as detailed below, and writing the manuscript.
- Linnea Berg contributed to this study by isolating and sequencing full-length *Xenopus* FOG cDNA.
- Jan L. Christian contributed to this study by generating FOG MO1, by subcloning the FOGΔNuRD and FOGΔCtBP constructs and by editing the manuscript. She also provided advice and suggestions on experimental design and data analysis, as well as the space, equipment, and supplies with which to perform the experiments.

Abstract

Friend of GATA 1 (*FOG-1*) is required for erythropoiesis in mammals and fish. By contrast, the finding that FOG inhibits red blood cell (RBC) development when overexpressed in *Xenopus* embryos suggests that it might normally repress erythropoiesis in this species. To definitively test this possibility, we used antisense morpholinos to knockdown expression of FOG in *Xenopus* embryos. We show that endogenous FOG is required for primitive erythropoiesis in frogs, and that it prevents excessive apoptosis of RBCs. To identify domains of FOG that are essential for blood development and, to begin to understand the mechanism(s) by which overexpressed FOG represses primitive erythropoiesis, we asked whether mutant forms of FOG that are unable to interact with known co-factors are able to rescue blood formation in FOG morphants and/or repress erythropoiesis when overexpressed in wild type embryos. We find that interaction of FOG with GATA factors and with the Nucleosome remodeling and deacetylase (NuRD) complex, but not with C-terminal binding protein, is essential for normal RBC development. By contrast, all of these interactions are dispensable for repression of erythropoiesis by overexpressed FOG. These data suggest that overexpressed FOG dominantly interferes with endogenous FOG function, and reveal a novel requirement for FOG-NuRD interaction during primitive erythropoiesis.

Keywords: Friend of GATA (FOG), GATA, *Xenopus laevis*, primitive hematopoiesis, erythropoiesis, CtBP, NuRD

Introduction

Vertebrate blood development takes place in two waves, called primitive and definitive hematopoiesis (reviewed in (Goldman and Christian, 2004). Primitive hematopoiesis is the initial wave of blood development in the embryo in which blood progenitor cells in the mesoderm give rise mainly to primitive red blood cells (RBCs), although some white blood cells are also produced at this time. By comparison, definitive hematopoiesis constitutes the second wave of blood development in which hematopoietic stem cells give rise to blood cells of all lineages. It begins later in development after primitive hematopoiesis and continues throughout adult life. Primitive hematopoiesis in mammalian embryos takes place extra-embryonically in blood islands of the yolk sac. Our studies take advantage of the much more accessible analogous structure in *Xenopus* embryos called the ventral blood island (VBI). The VBI is an intraembryonic structure that forms when cells from the rostral and caudal mesoderm converge during gastrulation and subsequently elongate along the ventral midline (Goldman and Christian, 2004; Lane and Smith, 1999). As RBCs form during the early tailbud stage, they begin to express the differentiation marker *globin*, which initiates in the anterior aspect of the VBI and extends posteriorly as more hemoglobin is synthesized. During the late tailbud stage (roughly stage 35-6), the heart begins to contract and RBCs begin circulating within the embryo (Zon, 1995).

The GATA family of zinc finger transcription factors plays an essential role during hematopoiesis. There are six vertebrate GATAs; GATA-1, -2 and -3 are

required for hematopoiesis, whereas GATA-4, -5, and -6 are required for cardiac, endoderm, gonadal and CNS patterning (Fujiwara et al., 1996; Tsai et al., 1994; reviewed in (Patient and McGhee, 2002). In the mouse, GATA-1 and GATA-2 play distinct, evolutionarily conserved roles during primitive erythropoiesis, which require FOG-1 as a cofactor. Targeted deletion of either *GATA-1 or GATA-2* in the mouse leads to death early in embryonic development from severe anemia (Fujiwara et al., 1996; Tsai et al., 1994). *GATA-1* null embryos have defects in primitive erythropoiesis, and RBC progenitor development is arrested at the proerythroblast stage (Fujiwara et al., 1996). Similarly, zebrafish embryos in which GATA-1 is mutated or depleted with morpholino oligos lack RBCs (Galloway et al., 2005; Lyons et al., 2002). Finally, GATA-1 is also required to support the viability of RBC precursors by suppressing apoptosis (Weiss and Orkin, 1995). By comparison, *GATA-2* null mice produce primitive erythrocytes that are morphologically normal but drastically reduced in number, resulting in embryos that also die *in utero* from anemia (Tsai et al., 1994). This finding supports *in vitro* data indicating that GATA-2 is not required for terminal erythroid differentiation, but is required to maintain the hematopoietic progenitor population (Tsai and Orkin, 1997). Likewise, loss of GATA-2 in zebrafish results in fewer total blood cells, consistent with its role in progenitor maintenance in mice (Galloway et al., 2005). Finally, mouse *GATA-1*/*GATA-2* double knockouts lack virtually all primitive erythropoiesis and thus display a more severe RBC defect than either of the single mutants (Fujiwara et al., 2004), indicating that GATA-1 and GATA-2 may be able to compensate for one another.

FOG-1 (Friend of GATA 1) functions as a critical transcriptional cofactor for both GATA-1 and GATA-2 during hematopoiesis. In the mouse, targeted deletion of FOG-1 results in a block in primitive erythropoiesis at the pro-erythroblast stage, phenocopying the RBC defect seen in *GATA-1* null mice (Tsang et al., 1998). Studies have also shown that a point mutation in GATA-1 that inhibits FOG-1 binding cannot rescue erythroid differentiation in a GATA-1 deficient cell line, and that this defect is rescued by co-expression of FOG-1 that bears a reciprocal mutation that restores binding (Crispino et al., 1999). The similarity in the FOG-1 and GATA-1 mouse knockout phenotypes together with *in vitro* mutant rescue data strongly suggests that they function together to promote RBC development. In addition, analogous human mutations that disrupt GATA-FOG interaction are associated with familial dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). While it is clear that FOG-GATA interaction is essential for RBC development, the mechanism by which FOG acts to promote RBC development is not well understood.

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X MSRRKQSNPR QIKRSLGDME GTEAKFIEEP NHSDKDGAYS DQDGSVDCDS PSPVNSDSNE
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H MSRRKQSNPR QIKRSLGDME AREEVQLVGA SHM..EQKAT APEA...... PSPPSADVNS

Z .......... .......... .......LYN EEPRTHESLA VSDEGEEDEK GLKRCTEDEE
X ENGCNSVTQS LEQESEEAAS KPSVELGQIT KSPCTSEGEL .....REDEE NIQESRSPSS
M .......... .......... .PAV...... PAPPESPEDP EDMEGQELEM RPQDEEKEEK
H .......... .......... .PPP...... LPPPTSPGGP KELEGQEPEP RP........

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M E..EEAAMAS PWSGPEELEL AL..QDGQRC VRARLSLTEG LSWGPFYGSI QTRALSPERE
H ...TEEEPGS PWSGPDELEP VV..QDGQRR IRARLSLATG LSWGPFHGSV QTRASSPRQA

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M EPGPAVTLM. VDESCWLRML PQVLTEEAAN SEIYRKDDAL WCRVTKVVPS GGLLYVRLVT
H EPSPALTLLL VDEACWLRTL PQALTEAEAN TEIHRKDDAL WCRVTKPVPA GGLLSVLLTA

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X EP..Q..AIP NYTI..KEEP GETSQCTSTL PEFQLLPQQA GMAAILATAV VNKDVFPCKD
M EP..H..GAP RHPV..Q.EP VEPGGLAPVH TDIQLLPQQA GMASILATAV INKDVFPCKD
H EP..H..STP GHPV..KKEP AEPTCPAPA. HDLQLLPQQA GMASILATAV INKDVFPCKD

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H CGIWYRSERN LQAHLLYYCA SRQGTGSPAA AATDEKPKET YPNERVCPFP QCRKSCPSAS

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M QHSLMHS..P LVPADK..AP TPSSGLDSK. ..AE...V.. ....TNGETR VPP..QNGGS
H QHTALQG..P LASADLGLAP TPSPGLDRK. ALAE...A.. ....TNGEAR AEPLAQNGGS

Z ASQGYSPLS. QLNIKEEPRS DYENDAKEEE Q.VNSPQENA AEASSSQPTS PKSPTVVAVK
X ASSRSEETPL KLYIKQEPE. ........GQ LSISEAGSTT CEAKDGVA.L VQSPA.IKVK
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H SEPPAAPRSI KVEAVEEPE. ........AA P.ILGPGEPG PQAPSRTP.S PRSPAPARVK
```

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H AELSSPTPGS SPVPGEL... .GLAGALFLP QYVFGPDA.. .....APPAS EILAKMSELV

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M HNRLQQGAGS SGAA...GTP TGLFSG.TKG ATCFECEITF NNINNFYVHK RLYCSGRRAP
H HSRLQQGAGA G.AG...GAQ TGLFPGAPKG ATCFECEITF SNVNNYYVHK RLYCSGRRAP

Z GETGGLVKEG AVTAAAPPAS HAASPQARPV SRAASASPSC PDPAPGG... .....TASEP
X DEN..... ..... .....SSS ARKVKAL.PA RTALASGFSS TEQEASPPQE
M .ED..... ..... .....PPT VRRPKAA.TG PARAPAG... .....AAAEP
H .ED..... ..... .....APA ARRPKAP.PG PARAPPG... .....QPAEP

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X GVA.PTEST. ..PPSPHTLG R.VEAMA.LM PGLIPAPVMP SPSSSPDAVD GPIDLSKKPR
M AAGAPPPA.A GPAPVPVVPS PTAELPSSPR PGSASAGPAP ALSPSP.VPD GPIDLSKRPR
H AAGAPPPPPP GHAPAPESPR PGS.GSG... ...SGPGLAP ARSPGP.AAD GPIDLSKKPR

Z LRESQ.RKDS IS.ALPLSDY HKCTACSISF NSIENYLAHK TYYCPATTLQ PQTTEQLNRL
X LVAEAPVPSA AATVAPLADY HECTACRISF NSLESYLAHK KFSCPTAPLQ QKTIQQLQKV
M R....QSPDA PTALPALADY HECTACRVSF HSLEAYLAHK KYSCPAAPLR ..........
H R....PLPGA P..APALADY HECTACRVSF HSLEAYLAHK KYSCPAAPPP GA........

Z KRPASTSPKN RAVDQHSDSK V.....LQTG KTAA..VAHA VPGS.ESTPP HVQGA....K
X KSPSSATGK. .LVDDTVKVK VESKAALSPG SVSETIQPLA LPFSTISDPK QLQQYSSVTE
M .......... .......... .......... .......... .......... ..........
H .......... .......... .......... .......... .......... ..........

Z TPSTSPVVCP YCPPNKLLTC DLMEHFKTTH GLVLTLQQ.H PETQSTGV.. ...SPSPSLS
X ASLSATTTCP YCPHNVIIRG DLLEHFRSVH GLILAKPTAG HRLQTTFMEV LVPARGQTSS
M ....TTALCP YCPPNGRVRG DLVEHLRQAH GLQVAKPAAS PGAEPR.... .TPA......
H .LGLPAAACP YCPPNGPVRG DLLEHFRLAH GLLLGAPLAG PGVEAR.... .TPA......

Z PREGAPLTP. PK........ PSSRPRKDSL NGRRIKLEAT SPSPPVLNGS SLESVGSRSP
X ASENSLPSPP .VSSASPLQL PGLRRENSNF KDTT.SSSSS ANGSPILTST PRPLL...PT
M .E..... ..... ...RAPRDSP DG..... ..RAPRSPSP APENT...PS
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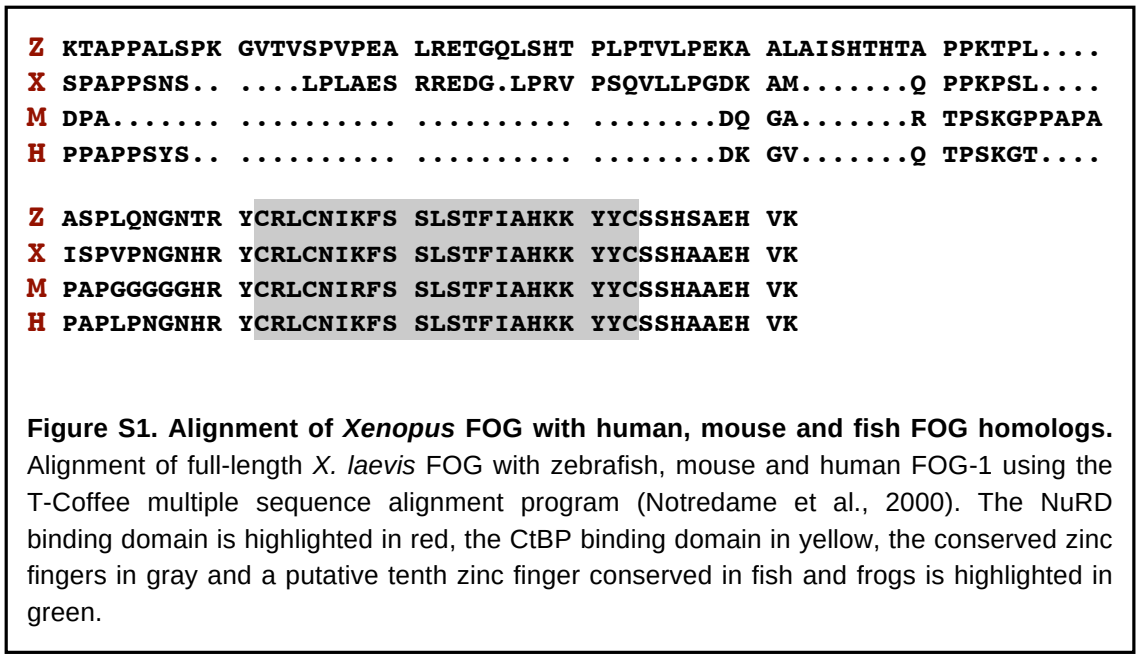


Figure S1. Alignment of *Xenopus* FOG with human, mouse and fish FOG homologs. Alignment of full-length *X. laevis* FOG with zebrafish, mouse and human FOG-1 using the T-Coffee multiple sequence alignment program (Notredame et al., 2000). The NuRD binding domain is highlighted in red, the CtBP binding domain in yellow, the conserved zinc fingers in gray and a putative tenth zinc finger conserved in fish and frogs is highlighted in green.

FOG serves as both a transcriptional co-activator and co-repressor of GATA target genes but the mechanisms by which it does so are unclear. FOG is a large multi-domain protein that includes nine conserved zinc fingers, four of which mediate GATA binding (Fox et al., 1998; Fox et al., 1999). There are two FOG homologs in the mouse, mFOG-1 and mFOG-2. Although they appear to be functionally redundant (e.g. forced expression of FOG-2 can rescue erythroid maturation in a *FOG-1*$^{-/-}$ cell line Chang et al., 2002) their non-overlapping patterns of expression enforce interaction with different GATA subfamilies, and result in distinct *in vivo* functions. Specifically, FOG-1 interacts with GATA-1, -2 and -3 and is involved in hematopoiesis, while FOG-2 interacts primarily with GATA-4, -5 and -6 (reviewed in Cantor and Orkin, 2005). Specific domains of FOG that function as transactivators, or that recruit accessory proteins to fulfill this role, have not been identified. By contrast, FOG has been shown to recruit

the transcriptional co-repressors C-terminal binding protein (CtBP) and members of the Nucleosome remodeling and deacetylase (NuRD) complex, although the functional significance of these interactions is not fully understood. Both co-repressor binding domains are highly conserved across evolution in a majority of FOG isoforms (Fig. 2-S1), and both co-repressors have been shown to interact with FOG *in vitro*, though only interaction with NuRD has been shown to be physiologically relevant *in vivo* thus far (Fox et al., 1999; Katz et al., 2002; Walton et al., 2006). The NuRD binding domain encompasses the first 12-14 residues at the N-terminus of FOG. This domain has been shown to be required for FOG function during heart and T-cell development (Kurata et al., 2002; Walton et al., 2006). Here, we have identified the presence of a NuRD binding domain in *Xenopus* FOG and investigate its role during primitive erythropoiesis.

While an essential role for FOG-1 during hematopoiesis has been well documented in mice and fish, the role of *Xenopus* FOG (xFOG) in this process is less clear. Only one FOG isoform has been identified in *Xenopus*. It is co-expressed with, and thus predicted to interact with all six GATA factors. Previous studies have shown that overexpression of wild type FOG suppresses erythropoiesis, whereas overexpression of FOG harboring a mutation that disrupts interaction with CtBP enhances primitive erythropoiesis in *Xenopus* embryos. These findings are consistent with the possibility that endogenous FOG functions to repress genes that are essential for RBC development via recruitment of CtBP (Deconinck et al., 2000). More recent studies have shown that downregulation of FOG is essential for commitment to certain non-erythroid

hematopoietic lineages (eosinophil and mast cell lineages), and that prolonged or ectopic expression can disrupt FOG-independent functions of *GATA* genes and prevent differentiation of these cell types (Sugiyama et al., 2008). These studies demonstrate that distinct levels of FOG expression are required in different developmental scenarios. Thus, the loss of RBCs observed upon overexpression of FOG in *Xenopus* may indicate that FOG negatively regulates erythroid development in this species, similar to what is observed for eosinophils or mast cells. Alternatively, loss of blood may be a secondary consequence of deregulating the proper balance between FOG and its other binding partners.

To definitively test the role of FOG in *Xenopus* erythropoiesis, we analyzed blood formation in embryos in which expression of FOG was reduced using antisense morpholino oligonucleotides. We find that in *Xenopus*, as in mice and fish, FOG is required for primitive erythropoiesis and specifically for RBC survival. To identify domains of FOG that are essential for this process and, conversely, to begin to understand the mechanism(s) by which overexpressed FOG represses primitive erythropoiesis, we asked whether mutant forms of FOG that are unable to interact with known co-factors were able to rescue blood formation in FOG morphants and/or repress blood development when overexpressed in wild type embryos. We find that interaction of FOG with GATA factors and the NuRD complex, but not with CtBP, is essential to support normal RBC development, but that all of these interactions are dispensable for repression of erythropoiesis by overexpressed FOG. These data suggest that overexpressed FOG dominantly interferes with endogenous FOG function, and

reveal a novel requirement for FOG-NuRD interaction during primitive erythropoiesis.

Materials and Methods

Embryo culture and manipulation

Ovulation was induced in adult *Xenopus laevis* females by injection of 50 IU of human chorionic gonadotropin (Sigma) into the dorsal lymph sac to induce spawning the night before egg collection. Embryos were staged according to Nieuwkoop and Faber (Moon and Christian, 1989; Nieuwkoop and Faber, 1994). Capped synthetic mRNA was synthesized by *in vitro* transcription of linearized template cDNA using a MegaScript kit (Ambion) and injected into the two vegetal blastomeres on the ventral side of eight-cell embryos to target prospective blood forming cells, as described previously (Moon and Christian, 1989).

Morpholinos and cDNA constructs

Sequence encoding the N-terminus of *Xenopus FOG* was obtained by 5' RACE using oligonucleotide primers complementary to sequence near the 5' end of the published partial length *xFOG* cDNA (accession no. **AF241228**). RNA was isolated from *Xenopus* embryos at stage 34, reverse transcribed and used as a template for PCR-mediated amplification of a *xFOG* cDNA (GenBank **GU384581** number -*submitted*-) that encodes the entire open reading frame, as determined by alignment with *FOG* from other species (Supp. Fig. 2-S1). A closely related cDNA that most likely represents a duplicate copy of the *xFOG* gene was

identified by searching The Gene Index Project database (Accession number TC411807). Morpholino antisense oligonucleotides complimentary to one or both alleles of *xFOG* (xFOGa: 5'-ATTGCTCTGTTTCCTTCTGGACATG-3' and xFOGb: GCTGGAGGACAAGGCAGGATCAAGC) were purchased from Gene Tools, LLC (Philomath, OR). Equal amounts of the two FOG MOs were mixed and the dose was titrated to 40ng per embryo.

Sequence encoding a MYC epitope tag was appended to the 5' end of the *xFOG* open reading frame by PCR-mediated amplification. Silent mutations to prevent morpholino annealing were engineered using a QuikChange XL (Stratagene) mutagenesis kit to generate a FOG rescue construct (xFOGr) with the following sequence at the 5' end: 5'*ATG* **GAA CAA AAA CTT ATT TCT GAA GAA GAT C**<u>**TG**</u> TC***T*** AGA ***C***GG AA***G*** CAG AG***T*** AA***C*** CCC AGA CAG. MYC tag is in bold, morpholino 2 target sequence is underlined, translation start site is italicized and silent mutations are bold and italicized. xFOG or xFOGr was used as a template to generate cDNAs encoding xFOGΔCtBP and xFOGΔNuRD by PCR-mediated mutagenesis. xFOGΔCtBP has a two residue substitution that converts the CtBP binding motif from PIDLSK to PIASSK. This mutation has been shown to disrupt binding of CtBP to mouse FOG-2 (Deconinck et al., 2000) and FOG-1 (Katz et al., 2002). xFOGΔNuRD lacks amino acids two through twelve, which have been shown to be essential for NuRD binding (Hong et al., 2005). The xFOGΔNuRD/ΔCtBP double mutant was generated by subcloning the CtBP substitution into the xFOGΔNuRD construct using restriction enzymes. xFOG4ZM was generated by using a QuikChange XL mutagenesis kit

(Stratagene). xFOG4ZM harbors a single tyrosine to alanine substitution in each of the four GATA-binding Zinc fingers (fingers 1,5,6 and 9) (Fox et al., 1999). Introduction of analogous point mutations into mouse FOG-1 has been shown to be sufficient to inhibit GATA binding (Cantor et al., 2002). All cDNAs were subcloned into pCS2+ for RNA transcription and transient transfection of mammalian cells.

Analysis of RNA

Total RNA was isolated and Northern blots were hybridized with antisense riboprobes as described previously (Christian et al., 1990; von Dassow et al., 1993). Bands were visualized with a phosphorimager and quantified using the NIH ImageJ software. Embryos were processed for *in situ* hybridization according to the protocol outlined in (Sive et al., 2000).

Immunoprecipitation and Western blot analysis

Protein was harvested from *Xenopus* embryos by freon extraction. Briefly, embryos were lysed in 15 mM Tris-Cl and an equal volume of 1,1,2-trichlorotrifluoroethane (freon; Sigma) with Compleat Mini protease inhibitor (Roche). Aqueous and organic phases were separated by microcentrifugation for 10 minutes at 4° C. xFOG and xFOG mutant expression vectors were generated by subcloning full-length *Xenopus* FOG into $pCS2^{+}$. The xGATA-2HA expression vector has been described previously (Dalgin et al., 2007). HeLa cells were transfected with 0.5 μg xGATA-2HA and 5.0 μg wild type or mutant xFOG-MYC

using lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. Cells were lysed after 24 hours and protein extracts prepared as described previously (Crossley et al., 1995). Protein extracts were incubated in 750 μL binding buffer (Tsang et al., 1997) containing 1:500 anti-MYC antibody (9B11; Sigma) at 4° C overnight. Complexes were precipitated with Protein G conjugated sepharose beads (Invitrogen) at 4° C for three hours, then washed four times in binding buffer. Proteins were denatured by boiling in loading buffer with 8% SDS and 5% β–mercaptoethanol, separated by 7% or 8% SDS-PAGE and transferred onto PVDF membrane. Membranes were probed with anti-HA (3F10; Roche, 1:1000), anti-MYC (71D10; Cell Signaling, 1:500) and anti-actin (Sigma, 1:10,000) antibodies and detected by chemiluminescence (Pierce).

Collection and analysis of peripheral blood samples

Tails were severed from tadpoles and blood was collected into medium containing 0.7X PBS, 0.5% BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytospin and stained with the Hema 3 stain set (Fisher Diagnostics). A minimum of 20 embryos was bled per experimental group and for each embryo the number of cells present in four random fields was counted at 20X magnification. Each experiment was repeated a minimum of three times and results were pooled.

TUNEL assays

Blood from stage 42 embryos was collected onto slides as described above and assessed for apoptosis using the DeadEnd Fluorometric TUNEL kit (Promega). Nuclei of cells were counterstained with propidium iodide to determine total numbers of cells. A minimum of 20 embryos was bled per experimental group and for each embryo the number of apoptotic and total cells present in four random fields was counted at 20X magnification. Each experiment was repeated a minimum of three times and results were pooled.

Results and Discussion

FOG is required for primitive red blood cell development in Xenopus

To determine whether FOG is required for primitive erythropoiesis in the frog, we designed anti-sense MOs (illustrated in Fig. 2-1A) capable of blocking translation of endogenous *x*FOG mRNA. RNA encoding MYC-epitope tagged xFOG (xFOG-MYC) was injected alone, or together with FOG MOs into *Xenopus* embryos at the two-cell stage, and expression of xFOG was examined by Western analysis of gastrula stage embryonic extracts using antibodies specific for the MYC tag. Levels of xFOG-MYC protein translated from a wild type RNA were reduced in the presence of the MO whereas there was no effect on translation of a rescue mRNA (xFOGr-MYC) containing silent mutations that prevent annealing of the MOs (Fig. 2-1B). These results show that the MOs specifically target wild type xFOG but not xFOGr mRNA. FOG MOs were then injected into both ventral vegetal blastomeres of eight-cell embryos (illustrated in Fig. 2-1C), as these cells are fated to give rise to most of the blood-forming

mesoderm (Dale and Slack, 1987; Dale et al., 1985; Lane and Smith, 1999). Following injection, embryos were cultured to the tailbud stage (st. 35-7) and examined for changes in expression of the RBC differentiation marker *globin,* by whole-mount *in situ* hybridization. MO-mediated knockdown of FOG resulted in a dose-dependent reduction in *globin* expression along the length of the ventral blood island, the primary site of primitive erythropoiesis in frog embryos (Fig. 2-1D). The loss of *globin* expression caused by injection of FOG MOs was confirmed by Northern blot analysis (Fig. 2-1E). We observed a significant reduction in *globin* expression when either the 40 ng or 60 ng dose of FOG MO was injected (Fig. 2-1D, E), and all subsequent experiments were performed using the lower dose. Injection of 200 pg of xFOGr RNA alone had no effect on expression of *globin*, whereas co-injection of the same dose of xFOGr together with FOG MOs significantly rescued loss of *globin* expression in morphants (Fig. 2-1F), showing that the loss of *globin* expression is specifically due to knock-down of FOG (Fig. 2-1F). These data demonstrate that FOG is required for primitive erythropoiesis in frogs.

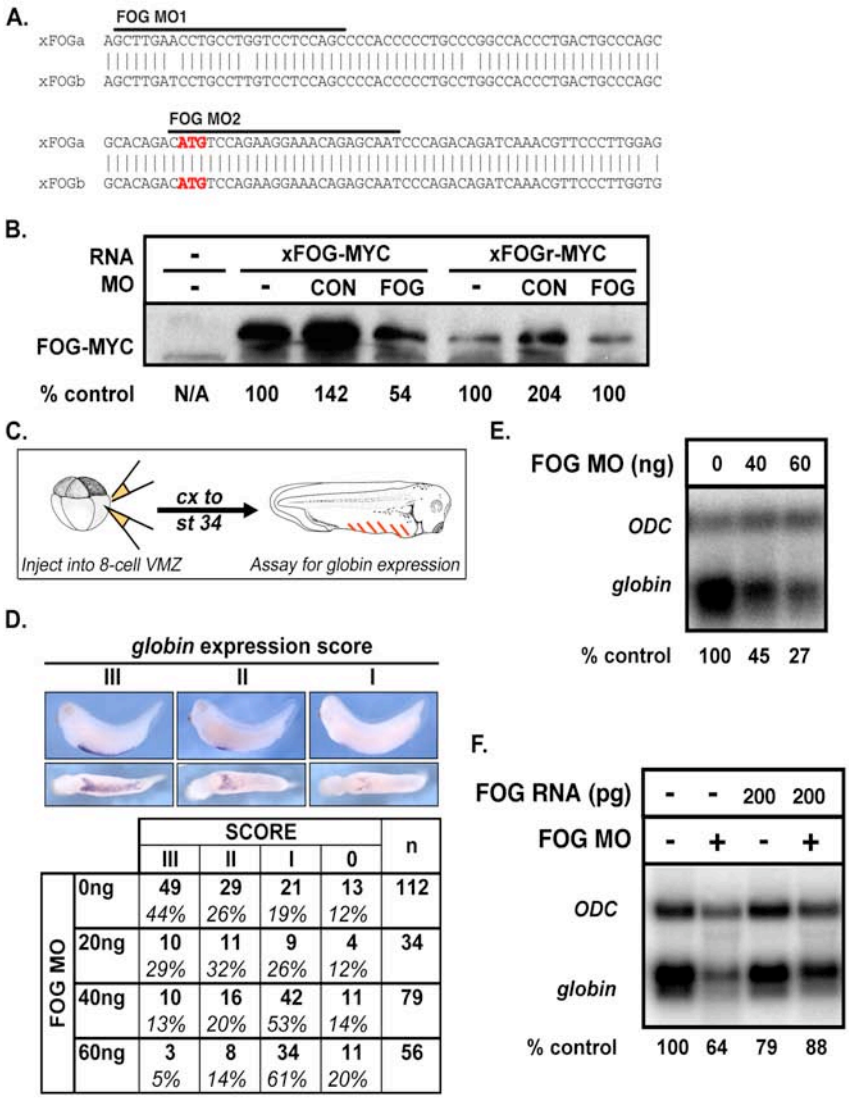


Figure 2-1. FOG is required for primitive erythropoiesis in *Xenopus laevis*. (A) Alignment of sequence surrounding the translation start site of the two *Xenopus FOG* alleles. Sequences to which FOG MOs bind are indicated by he black bars and the ATG start codon is in red. (B) Anti-MYC Western blot of lysates from embryos injected with FOG MO and either wild type xFOG-MYC or xFOGr-MYC RNA, which harbors silent mutations that prevent MO binding. Note that FOG MOs can block the translation of xFOG-MYC mRNA but do not affect translation of xFOGr-MYC RNA *in vivo*. Levels of protein expressed from xFOG-MYC or xFOGr-MYC are normalized to the RNA-only injected control for each construct. (C) Illustration of MO and/or RNA injection near the ventral marginal zone (VMZ) of an eight-cell embryo. Injected embryos are cultured to the tailbud stage and assayed for *globin* expression in the VBI (depicted in red) by *in situ* hybridization (D) or Northern blotting (E,F). (D) *In situ* hybridization analysis of *globin* expression (purple stain) in embryos injected with increasing doses of FOG MOs. (E,F) Northern blot of *globin* expression at stage 34 in embryos injected with increasing doses of FOG MO (E) or injected with FOG MO (40 ng), FOG RNA (200 pg), or both (F). Levels of *globin* expression are normalized relative to expression of *ODC* and reported as a percentage of control below each lane. Similar results were obtained in a minimum of three independent experiments in all morphant analysis.

FOG morphants show a loss of circulating RBCs that is due to increased apoptosis

To better understand the mechanism by which loss of *globin* was occurring in our FOG morphants, we collected total circulating blood cells from individual embryos at the tadpole stage, concentrated them onto slides by cytocentrifugation and stained with a Wright-Giemsa differential stain to look for changes in RBC number and morphology. Compared to wild type sibling controls, the total number of RBCs in morphants was drastically reduced (Fig. 2-2A,B). In addition, FOG morphant RBCs were smaller in size, showed reduced cytoplasm and condensed nuclei relative to control RBCs (Fig. 2-2A, black arrows). In contrast, we did not note any reproducible differences between control and FOG morphant tadpoles in the number or morphology of white blood cells, which are predominantly of the monocyte/macrophage lineage at this stage of development (Fig. 2-2A, red arrow, data not shown). The nearly complete absence of RBCs in FOG morphants was striking, given the more modest reduction in levels of *globin* at earlier (tailbud, stage 35-7) stages of development (Fig. 2-1). When analyzed at the tadpole stage (stage 42), however, we observed a severe reduction in expression of *globin* that paralleled the dramatic loss of RBCs in morphants (Fig. 2-2C).

The morphology of RBCs isolated from FOG morphants is consistent with that of cells undergoing programmed cell death. To more stringently test this possibility, we performed TUNEL assays on circulating blood cells isolated from wild type and FOG morphant tadpoles. The significant reduction in the total

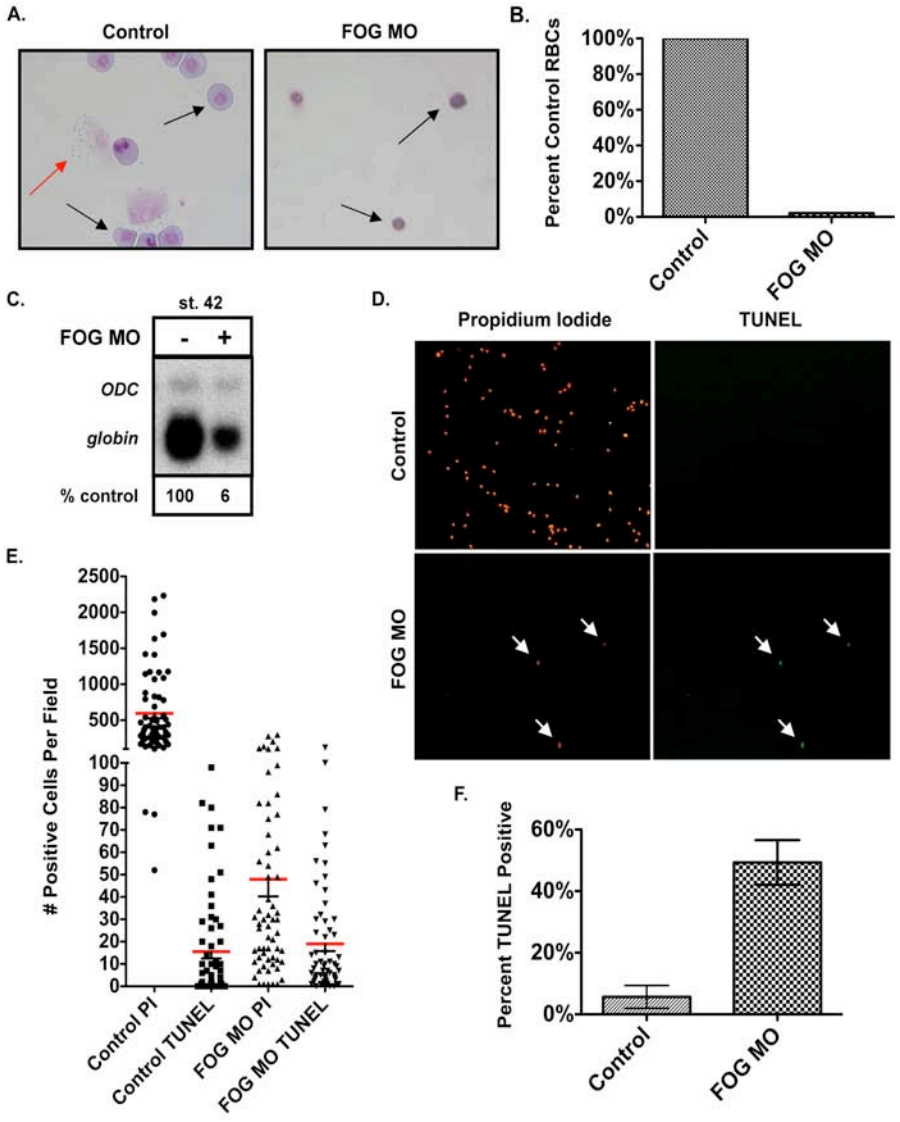


Figure 2-2. FOG morphants show a loss of circulating RBCs that is due to increased apoptosis. (A) Wright-Giemsa stain of blood cells from wild type and FOG MO injected embryos. Black arrows indicate primitive RBCs, red arrow indicates a primitive white blood cell. (B) Graph showing the number of circulating RBCs in tadpoles that had been injected with FOG MOs at the eight-cell stage as compared to their wild type siblings. The number of RBCs is expressed as a percentage of control +/- SEM. (C) Northern analysis of *globin* expression in stage 42 uninjected control and FOG MO injected embryos. Levels of *globin* expression are normalized relative to expression of *ODC* and reported as a percentage of control below each lane. (D) TUNEL staining of circulating blood cells. White arrows indicate cells that are both propidium iodide and TUNEL positive. (E) Scatter plot showing the number of propidium iodide (PI) and TUNEL positive cells per high-power field in blood from uninjected control and FOG MO injected embryos. Red bars indicate the mean. (F) Graph showing the number of TUNEL positive cells as a percentage of total (propidium iodide positive) cells in uninjected control and FOG MO injected embryos. All results were reproduced in a minimum of three independent experiments.

number of blood cells present in FOG morphants was accompanied by an increase in the fraction of cells that were TUNEL positive. (Fig. 2-2D and E, and as quantified in Fig. 2-2F). These observations indicate that FOG is required for survival of mature RBCs, and that in the absence of FOG, RBCs undergo excessive apoptosis.

The finding that FOG is required for survival of RBCs is consistent with previous *in vitro* data demonstrating that in the absence of GATA-1, RBCs undergo excessive apoptosis (Weiss and Orkin, 1995). In addition, GATA-1 has been shown to specifically activate genes involved in mediating RBC survival (Gregory et al., 1999; Maeda et al., 2009). Our data demonstrating that primitive erythrocytes undergo apoptosis in the absence of FOG, together with data from others, suggest that GATA-1 and FOG function together to promote expression of anti-apoptotic genes in order to support RBC survival.

Overexpression of FOG inhibits erythropoiesis independent of interactions with CtBP, the NuRD complex, or GATA factors

As described in the introduction, previous studies in *Xenopus* have found that overexpression of wild type FOG represses RBC development, but that a mutant form of mouse FOG-2 that cannot bind CtBP enhances RBC development in *Xenopus* embryos (Deconinck et al., 2000). This is consistent with the possibility that endogenous xFOG normally functions to block RBC development by repressing genes required for erythrocyte maturation via recruitment of CtBP. These findings, however, are in contrast to published data

showing that CtBP binding is dispensable for mFOG-1 function in murine erythropoiesis (Katz et al., 2002), and to our current data, which demonstrate that FOG is required to support *Xenopus* erythropoiesis.

Given the above inconsistencies, we wished to test the possibility that the loss of blood seen upon overexpression of FOG instead reflects an imbalance between endogenous FOG and its binding partners. We also wished to ask which binding partners, if any, are required to produce the overexpression phenotype. To begin to address these questions, we analyzed blood development in embryos made to express MYC-epitope tagged wild type and mutant forms of xFOG (illustrated in Fig. 2-3A). RNA encoding wild type xFOG (50 pg to 1 ng) was injected near the ventral midline of four-or eight-cell embryos and expression of *globin* was analyzed at the tailbud stage by Northern blotting. Doses of xFOG RNA of 300 pg or greater reproducibly resulted in a 40-50% reduction in levels of *globin* expression (data not shown), whereas doses of xFOG RNA less than or equal to 200 pg resulted in no significant reduction in levels of *globin* expression (Fig. 2-1F). To determine whether the ability of overexpressed FOG to cause a loss of blood requires interaction with known binding partners, we analyzed *globin* levels in embryos made to express FOG containing point or deletion mutations (illustrated in Fig. 2-3A). These mutants have been shown by others to prevent interaction with CtBP (Katz et al., 2002) (xFOGΔCtBP), the NuRD repressor complex (Hong et al., 2005) (xFOGΔNuRD), and/or GATA factors (Fox et al., 1998) (xFOG4ZM) (see materials and methods for details). xFOGΔCtBP and xFOG4ZM contain silent mutations that prevent

annealing of the FOG MOs and xFOGΔNuRD lacks the MO recognition sequence altogether due to its N-terminal deletion. Steady state levels of all three of these FOG mutants were roughly equivalent to that of wild type FOG following transient transfection into HeLa cells, as analyzed by Western blotting (Fig. 2-3B), demonstrating that the mutations do not affect protein stability. In addition, co-immunoprecipitation assays revealed that interaction of xFOG4ZM with GATA-2 was severely impaired relative to interaction with wild type xFOG, xFOGΔNuRD or xFOGΔCtBP (Fig. 2-3C). The xFOG4ZM mutant was unable to rescue blood formation in FOG morphants, consistent with previous studies showing that FOG-GATA interactions are essential for RBC development (Fig. 2-3D) (Crispino et al., 1999; Nichols et al., 2000). This result confirms that the xFOG4ZM mutant is defective in binding endogenous GATA proteins, even when overexpressed in the embryo. We then injected 500 pg of RNA encoding either wild type or mutant forms of FOG into the two ventral vegetal blastomeres of eight-cell embryos and analyzed expression of *globin* by Northern blotting. In contrast to previous findings that overexpression of mouse FOG-2ΔCtBP in *Xenopus* embryos resulted in increased RBCs, and elevated levels of hemoglobin (Deconinck et al., 2000), we found that overexpression of xFOGΔCtBP caused a reduction in *globin* expression (Fig. 2-3E). A similar reduction in *globin* expression was observed in embryos made to overexpress xFOG4ZM, xFOGΔNuRD, or xFOGΔNuRD/ΔCtBP, which is unable to interact with either CtBP or the NuRD complex (Fig. 2-3E).

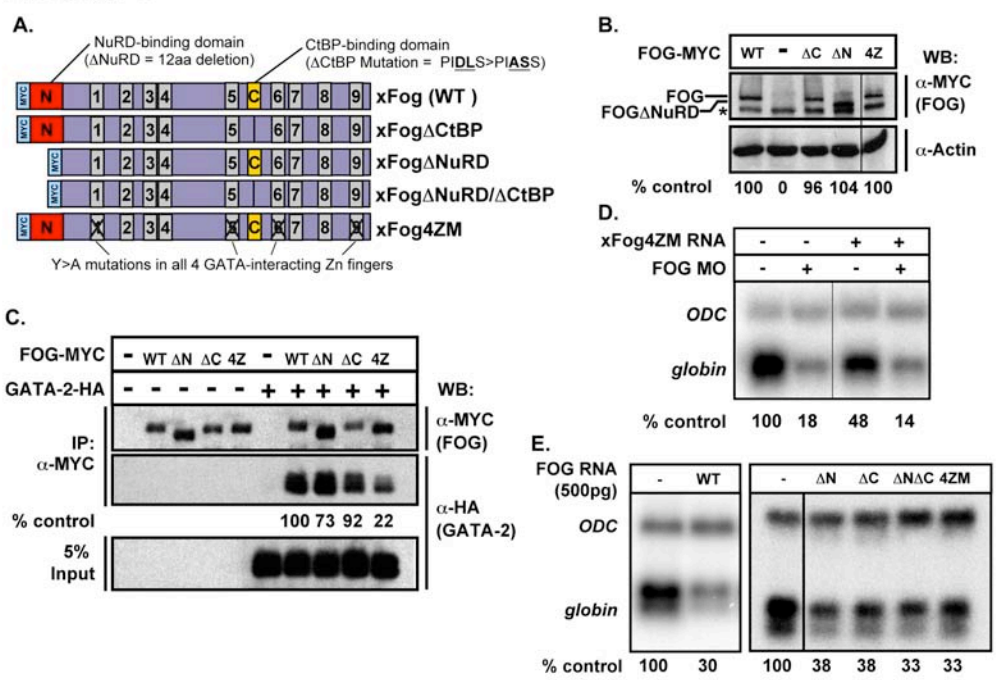


Figure 2-3. Overexpression of FOG or mutant forms of FOG inhibits normal erythropoiesis. (A) Schematic of wild type and mutant xFOG-MYC isoforms. The MYC tag is in blue, the NuRD binding domain in red, the CtBP binding domain in yellow and the zinc fingers are in grey. (B) Western analysis of cell lysates from HeLa cells transiently transfected with MYC epitope-tagged wild type and mutant xFOG cDNA constructs. Levels of FOG protein expression are normalized to expression of Actin and reported as a percentage of the wild type control below each lane. Asterisk indicates a non-specific background band that is observed even in the absence of FOG. (C) Wild type and mutant MYC epitope-tagged xFOG constructs were transfected into HeLa cells alone, or together with HA-tagged GATA-2. Western blots (WB) of cell lysates (input) or anti-MYC immunoprecipitates (IP) were probed with an anti-HA antibody to detect interaction with GATA-2-HA. Levels of GATA-2 protein detected by co-IP are normalized to the GATA-2 input and to the FOG IP and reported as a percentage of the wild type control below each lane. (D) FOG MO (40 ng) and xFOG4ZM RNA (200 pg) were injected alone or together into the ventral vegetal blastomeres of eight-cell embryos and relative expression of *globin* was determined by Northern blotting at stage 34. (E) RNA (500 pg) encoding wild type or mutant xFOG was injected into the ventral vegetal blastomeres of eight-cell embryos and expression of *globin* was analyzed by Northern blotting at stage 34. Levels of *globin* transcripts are normalized relative to expression of *ODC* and reported as a percentage of control below each lane. Results were reproduced in a minimum of three independent experiments. (ΔN=xFOGΔNuRD, ΔC=xFOGΔCtBP, ΔNΔC= xFOGΔNuRD/ΔCtBP, 4Z = xFOG4ZM)

Interestingly, our data show that both down- and up-regulation of FOG in *Xenopus* embryos disrupts primitive erythropoiesis. Preliminary bleeds from

tadpoles that overexpress wild type or mutant FOG isoforms indicate that they also exhibit reduced RBC numbers (data not shown). One possible explanation for this phenomenon is that excess FOG creates a squelching effect by which other limiting transcriptional co-factors are sequestered away from their endogenous target promoter(s). Another possibility is that overexpressed FOG may be prematurely facilitating GATA-1/FOG induced repression at genes required for RBC progenitor expansion such as *GATA-2* (Grass et al., 2003; Weiss et al., 1994) and *Kit* (Jing et al., 2008), and thereby limiting the number of precursors available to differentiate as RBCs. This latter hypothesis seems less likely in light of the fact that overexpression of both wild type FOG and mutant forms of FOG which lack the ability to interact with all known repressor complexes resulted in the same dramatic reduction in *globin* expression. Instead, we favor the former hypothesis, which predicts that very precise regulation of FOG protein levels is important for proper blood development. Due to lack of an adequate *Xenopus* FOG antibody, we were unable to measure endogenous FOG levels. However, using *globin* expression as a functional readout of successful RBC development, we find that the ability of FOG to repress erythropoiesis is only observed once a certain threshold dose of FOG has been exceeded. Moreover, this effect persists regardless of the isoform of FOG overexpressed, including xFOG4ZM. Because this mutant cannot interact with GATA and is unable to rescue endogenous RBC development in FOG morphants, we would not expect it to have a physiologically relevant role during RBC development. We therefore predict that loss of blood upon overexpression of FOG is likely due to a

dominant negative effect resulting from supraphysiologic levels of FOG. Consistent with this hypothesis, a recent study has emerged demonstrating that Mta3, a component of the NuRD complex, is required for primitive erythropoiesis (Li et al., 2009). Mta3 is likely recruited to blood targets through its association with FOG. Based on these findings and our above data, we would predict that overexpressed FOG may block Mta3 function by sequestering it and other components of the NuRD complex away from endogenous targets, thereby inhibiting erythrocyte development. As overexpression of xFOGΔNuRD also results in loss of blood, it is probable that other binding partners besides those of the NuRD complex are similarly prevented from executing their normal functions. We conclude that precise regulation of FOG levels in the embryo is crucial for normal blood development and that, consistent with our hypothesis, excess FOG inhibits blood development independent of its ability to recruit CtBP, the NuRD complex or GATA factors.

The CtBP binding site of FOG is dispensable, whereas the NuRD binding domain is required for FOG function in primitive erythropoiesis

The above results demonstrate that overexpressed FOG can dominantly interfere with normal erythropoiesis independent of its ability to recruit CtBP or the NuRD repressor complex. We next wished to determine whether recruitment of these factors is essential for endogenous FOG function during blood development. To do so, we asked whether mutant forms of FOG that are unable to interact with CtBP or the NuRD complex can rescue loss of blood in FOG

morphants. FOG MOs were injected into the two ventral vegetal blastomeres of eight-cell embryos either alone, or together with RNA (200 pg) encoding xFOGΔCtBP or xFOGΔNuRD and expression of *globin* was analyzed at the tailbud stage. Injection of 200 pg of RNA encoding xFOGΔCtBP had no effect on expression of *globin* in wild type embryos and was able to significantly restore globin expression in FOG morphant embryos (Fig. 2-4A). This suggests that direct FOG-CtBP interaction is dispensable for primitive erythropoiesis in frogs.

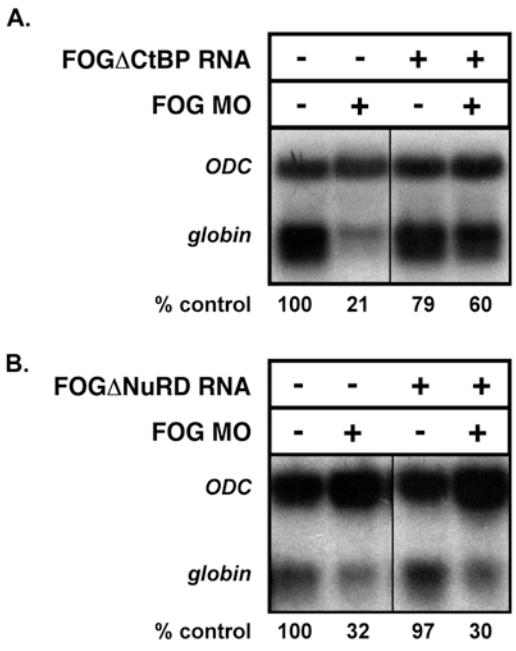


Figure 2-4. FOG interaction with NuRD but not CtBP is required for FOG function in erythropoiesis. FOG MO and 200pg of xFOGΔCtBP RNA (A) or xFOGΔNuRD RNA (B) were injected alone or together into the ventral vegetal blastomeres of eight-cell embryos and expression of *globin* was analyzed by Northern blotting at stage 34. Levels of *globin* transcripts are normalized relative to expression of *ODC* and reported as a percentage of control below each lane. Similar results were obtained in a minimum of three independent experiments.

By contrast, injection of 200 pg of RNA encoding xFOGΔNuRD also had no effect on *globin* levels when injected alone into wild type embryos, but did not rescue loss of *globin* in FOG morphants (Fig. 2-4B). The failure of xFOGΔNuRD to rescue RBC formation in FOG morphants uncovers a novel requirement for FOG-NuRD interaction during primitive erythropoiesis.

Our finding that the CtBP binding site of FOG is dispensable for FOG

function in primitive erythropoiesis is in agreement with gene targeting studies in the mouse which demonstrated that substitution of *mFOG-1ΔCtBP* at the endogenous *mFOG-1* locus did not disrupt erythropoiesis, even under conditions of erythropoietic stress (Katz et al., 2002). Together, these studies suggest that the CtBP binding domain is not required for normal primitive erythropoiesis in vertebrates. It remains possible, however, that CtBP is independently recruited to FOG by other nucleating factors that can themselves interact directly with FOG. Precedence for this type of cooperative interaction between multiple binding partners, in which elimination of a single binding site does not interfere with functional complex assembly *in vivo*, exists, for example, in the case of the axin destruction complex during Wnt signal transduction (Peterson-Nedry et al., 2008). This type of association would not require an intact CtBP binding site on FOG, and would not be detected when analyzing the ability of recombinant FOG and CtBP to co-immunoprecipitate *in vitro*, as other endogenous interaction partners would be absent. Chromatin immunoprecipitation to ascertain whether or not CtBP occupancy changes at blood-specific genes regulated by FOG/GATA in CtBP binding mutants (e.g. in FOG morphant embryos rescued with xFOGΔCtBP or in the *mFOG-1ΔCtBP* mutant mouse) relative to wild type controls would be one way to definitively address this question *in vivo*.

In contrast to our finding that the CtBP binding site on FOG is not required for normal RBC development, our results indicate that the NuRD domain is essential for this function. This was a bit surprising given previous studies showing that in a *FOG-1* null cell line, mutant forms of FOG lacking large portions

of the N-terminus encompassing and extending beyond the NuRD binding domain are able to rescue erythroid differentiation to the same extent as wild type FOG (Cantor et al., 2002). It is likely that functional differences between cell lines and *in vivo* differentiation account for this discrepancy. By contrast, our finding, that the NuRD domain is required for primitive blood development, is consistent with FOG function in other developmental contexts. For example, the NuRD binding domain has been shown to be both necessary and sufficient to mediate repression of GATA-4 targets in the heart (Lin et al., 2004; Svensson et al., 2000), and mutation of select residues which abrogate NuRD binding prevent normal heart development in zebrafish embryos (Walton et al., 2006). FOG-NuRD interaction has also been shown to be required during T-cell differentiation (Kurata et al., 2002). As we were preparing our report for submission, a complementary manuscript emerged demonstrating that mice carrying knock-in mutations at the *FOG-1* allele that abrogate NuRD binding show defects in definitive hematopoiesis. These defects phenocopy, but are milder than those observed in *FOG-1* null mutants (Miccio et al., 2009). These studies also reported the surprising finding that interactions with the NuRD complex are required for FOG-dependent activation, as well as repression, of GATA target genes. The requirement for NuRD interaction is thus a recurring theme in developmental processes that require FOG function, and we extend this observation to include primitive erythropoiesis.

Acknowledgements

We thank Linnea Berg for isolation and sequencing of the full-length *Xenopus* FOG cDNA and members of the Christian lab and J. Oyer for critical reading of this manuscript. This work was supported by a grant from the NIH (R03 HD050242) to JLC. MM was supported in part by a grant from the NIH (T32, HD049309) and a Tartar Trust Fellowship.

CHAPTER 3

Non-canonical Wnt signaling downstream of GATA-2 in the ectoderm represses canonical Wnt signaling to promote red blood cell commitment in the mesoderm during primitive erythropoiesis

Mizuho S. Mimoto, Devorah C. Goldman and Jan L. Christian

Department of Cell & Developmental Biology, Oregon Health & Science University. Portland, OR USA

PREFACE

This manuscript is currently in process.

- My contributions to this study include generating and collecting samples for the microarray analysis, guiding subsequent data analysis, target validation by qPCR and *in situ* hybridization (Figs 3S-1,3-2, 3-3A), Wnt11 MO knockdown experiments (Fig. 3-5B), and writing the manuscript.
- Devorah C. Goldman contributed to this study by performing ectoderm removal assays (Fig. 3-1).
- Carl Pelz contributed to this study by providing technical assistance with statistical analysis of the microarray data.
- Jenelle Johnston contributed to this study by sequencing and generating *in situ* probes for target validation.
- Jan L. Christian contributed to this study by performing microarray target validation by *in situ* hybridization (Figs. 3-3B-G, 3-4), non-canonical Wnt knockdown (3-5A), LiCl assays (3-6) and by editing the manuscript. She also provided advice and suggestions on experimental design and data analysis, as well as the space, equipment, and supplies with which to perform the experiments.

Abstract

Primitive erythropoiesis, which produces the first red blood cells in the embryo, is regulated in a non cell-autonomous fashion across evolution from frogs to mammals. In *Xenopus laevis*, signals from the overlying ectoderm are required to induce the mesoderm to adopt an erythroid fate. Previous studies in our lab identified the transcription factor GATA-2 as a key regulator of this ectodermal signal. To identify GATA-2 targets in the ectoderm that are required for red blood cell formation in the mesoderm, we used microarray analysis to compare ectoderm from GATA-2 depleted and wild type embryos. Our analysis identified the non-canonical and canonical Wnt pathways as being reciprocally regulated downstream of GATA-2. These results are consistent with a hypothesis in which non-canonical Wnt signaling is activated downstream of GATA-2 in the ectoderm, and that this in turn is required to inhibit the canonical Wnt pathway, thereby permitting red blood cell progenitors to exit the cell cycle and adopt a hematopoietic fate.

Key Words: GATA, Non-canonical Wnt, Canonical Wnt, Primitive Erythropoiesis, *Xenopus laevis*, Embryonic Development, Blood, Hematopoiesis

Introduction

Vertebrate hematopoiesis occurs in two spatially and temporally distinct waves, termed primitive and definitive hematopoiesis (reviewed in (Galloway and Zon, 2003; Goldman and Christian, 2004). Primitive hematopoiesis is the process by which mesodermally derived hematopoietic progenitor cells develop into a single lineage of mature blood cells. This initial wave of hematopoiesis primarily gives rise to primitive erythroid cells, which are the first red blood cells (Galloway and Zon, 2003; Goldman and Christian, 2004) in the early embryo. Also at this time, a subset of primitive myeloid cells of the macrophage lineage arise independently from a distinct population of mesodermal progenitors (Smith et al., 2002; Tashiro et al., 2006). Primitive erythropoiesis is a transient process that occurs extra-embryonically in the yolk sac blood islands in mammals, and intra-embryonically in the ventral blood island (VBI) during amphibian development (Ciau-Uitz et al., 2000). By contrast, definitive hematopoiesis initiates later in embryonic development, continues throughout adult life, and generates hematopoietic stem cells (HSCs), which can individually give rise to the full complement of blood cells from all lineages (Orkin and Zon, 2008).

Both primitive and definitive hematopoiesis are highly dependent on signals from non-hematopoietic cells in the surrounding tissue, or “niche,” for appropriate cues during development. During definitive hematopoiesis, the hematopoietic niche is composed of osteoblast cells that reside in bone marrow and send requisite signals to the HSC that promote a normal balance between stem cell self-renewal and differentiation (Calvi et al., 2003). The niche cells

create a microenvironment to support this balance between stem cell and end-organ maintenance, which is dependent on signaling through adhesion molecules and local signaling molecules such as Neural cell adhesion molecule (NCAM), BMP, Notch and Wnt (Baksh and Tuan, 2007; Iwasaki and Suda, 2009; Luis and Staal, 2009). For example, signaling via the Notch pathway promotes stem cell quiescence during phases of rest, whereas β-catenin-mediated Wnt signaling promotes stem and progenitor cell expansion and differentiation when new hematopoietic cells are needed for organ maintenance (Iwasaki and Suda, 2009; Luis and Staal, 2009).

Wnt pathway members have been shown to play a particularly important role in regulating the balance between HSC maintenance and differentiation (Sinenko et al., 2009; Staal and Luis, 2010). This is particularly evident in studies in which canonical, or β-catenin-mediated, Wnt signaling is constitutively activated in hematopoietic cells. Stem cells in these animals proliferate and expand rapidly resulting in their subsequent exhaustion and failure in long-term transplantation assays (Kirstetter et al., 2006; Scheller et al., 2006). By contrast, other studies have demonstrated that signaling through the non-canonical Wnt pathway inhibits proliferation through repression of canonical Wnt signaling and thus may have a tumor-suppressive effect. Mice that lack the non-canonical Wnt ligand, Wnt5a, show an increase in the incidence of certain canonical Wnt-associated leukemias, consistent with its proposed role as a tumor suppressor (Liang et al., 2003; Roman-Gomez et al., 2007).

Signals from the microenvironment, or niche, are also important for primitive erythropoiesis. In *Xenopus*, several lines of evidence indicate that the overlying ectoderm is the source of essential signals from non-hematopoietic cells, which are required to induce erythropoiesis in the mesoderm. First, when embryos are induced to exo-gastrulate by treatment with a high-salt solution such that the ectoderm and mesoderm do not come into contact during gastrulation, these embryos fail to express the erythrocyte differentiation marker, *globin* (Kikkawa et al., 2001). However, exogastrulae continue to express markers of other mesodermally-derived tissues, indicating that a requirement for ectodermal signals is unique to blood (Ruiz i Altaba, 1992). Second, studies by Maeno and colleagues demonstrated that when the ventral mesoderm is explanted at the gastrula stage and cultured to the tailbud stage, it does not form RBCs (Maeno et al., 1994b). However, when these explants are co-cultured with ectoderm from the same stage embryo, erythroid development is restored (Maeno et al., 1994b). Moreover, when the ectoderm is removed from embryos later in development during neurulation (stage 16), the mesoderm expresses *globin* at the tailbud stage, indicating that signals essential for blood development were transmitted from the ectoderm to the mesoderm prior to the mid-neurula stage (Maeno et al., 1994b). Similar studies in both chicken (Miura and Wilt, 1969) and mouse embryos (Belaoussoff et al., 1998) have also demonstrated an analogous requirement for inductive signals from non-hematopoietic cells during primitive erythropoiesis.

Recent work from our lab demonstrated that the transcription factor GATA-2 is required in the ectoderm in order for mesoderm to differentiate as blood (Dalgin et al., 2007). Specifically, ectoderm in which GATA-2 has been depleted by antisense morpholino oligonucleotide (MO) injection is no longer able to support blood formation when recombined with wild type mesoderm. In the current study, we have used microarray analysis to identify signaling molecules that are transcriptionally regulated by GATA-2 in the ectoderm, and are required for blood formation in the mesoderm. Our preliminary studies suggest that ectodermal GATA-2 activates non-canonical Wnt signaling, which is subsequently required to dampen canonical Wnt signals, which support progenitor maintenance in the mesoderm. This allows progenitor cells to exit the proliferative state and differentiate as primitive erythrocytes.

Materials and Methods

Embryo culture and manipulation

Ovulation was induced in adult *Xenopus laevis* females by injection of 50 IU of human chorionic gonadotropin (Sigma) into the dorsal lymph sac the night before egg collection in order to induce spawning. Embryos were staged according to Nieuwkoop and Faber (Moon and Christian, 1989; Nieuwkoop and Faber, 1994). Capped synthetic mRNA was synthesized by *in vitro* transcription of linearized template cDNA using a MegaScript kit (Ambion) and injected as indicated in the text and described previously (Moon and Christian, 1989). Ectoderm or mesoderm explants were dissected with watchmakers' forceps and

cultured independently or as recombinants in 0.5X normal amphibian medium (NAM/2; (Peng, 1991) as described previously (Goldman et al., 2006). Ectoderm removal assays were performed at the stages indicated in the text by removal of the dorsal half of the embryo using forceps and then using an eyebrow knife (Sive et al., 2000) to peel back the ectoderm. Transient inhibition of the canonical Wnt pathway was achieved by culture of whole embryos at the stages indicated with 0.25 M LiCl in 0.5X Modified Barth's Saline (MBS) for twenty minutes followed by 4-5 washes in 0.5X MBS.

Microarray Analysis

To generate samples for the microarray analysis, RNA or anti-sense morpholino oligonucleotides (MOs) were injected into both cells of the animal pole of embryos at the two-cell stage. Embryos were allowed to develop to stage 10, at which point ectoderm was removed using watchmakers' forceps and cultured to stage 12 in 0.5X NAM. Approximately 40 ectodermal explants from a single injection condition were pooled and RNA samples were generated for microarray analysis according to standard protocols provided by the manufacturer (Affymetrix). Briefly, RNA was extracted using Trizol (Invitrogen) and purified with an RNA clean-up kit (QIAGEN). RNA samples were sent to the OHSU Affymetrix Microarray Core facility for further processing and hybridization to the microarrays. Three chips were used for each of the four microarray condition using biological replicates collected during three separate days of experiments. Up to three females were used on each day to ensure that an

adequate number of eggs were spawned. Array probe levels were summarized using the Robust Multiarray Analysis RMA method (Irizarry et al., 2003a; Irizarry et al., 2003b), implemented in the Affymetrix package under BioConductor in R. A second level of probeset normalization was performed using the Global Rank-Invariant Set Normalization (GRSN) method (Pelz et al., 2008). Hierarchical clustering based on a Pearson's correlation coefficient was performed as an unsupervised method to identify outliers. Based on this analysis, Uninjected #3 was identified as a significant outlier and was removed from subsequent analysis. A batch adjustment was performed using an internal method based on Distance Weighted Discrimination (DWD) (Benito et al., 2004), which was developed by our statistician, Carl Pelz. This analysis was used to correct for a batch effect from the different biological replicates, likely due to the fact that *Xenopus laevis* is not isogenic, creating significant degree of background variability between the biological replicates that was unrelated to our scientific question. To facilitate analysis of a smaller sample size, the eBayes method (implemented through (Smyth, 2004) was used to identify up and down-regulated genes. Genes that showed changes +/- 1.2-fold were considered potentially significant.

Analysis of RNA

For Northern blotting, total RNA was isolated and Northern analysis was performed as described previously (Christian et al., 1990; von Dassow et al., 1993). Bands were visualized with a phosphorimager and quantified using the

NIH ImageJ software. For qRT-PCR analysis, total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions, from which cDNA was generated using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Inc.) with a poly d(T) primer. qRT-PCR was performed using a SYBR Green-based assay (QIAGEN) and a 7900 HT Sequence Detector (ABI). Each sample was analyzed in triplicate and normalized to ODC. Primers used are as follows: *ODC*: F 5′-TGC AGA GCC TGG GAG ATA CT-3′, R 5′-CAT TGG CAG CAT CTT CTT CA-3′; *xWnt11*: F 5′-TCC AGC GCA TGT CAG ATA AG-3′, R 5′-TGG AAT GAG AGC GAA CAC TG-3', *xEphrinB2*: F- 5′3′, R- 5′3, *xcdc42GEF*: F 5′-CCC AGA CAA GCT GAA GAA GG-3′, R 5′- CTG GTA CTG AAT CCG GCA AT-3, *xFz8*: F 5′-CAG AAC CTG GAC AAC CTG C-3′, R 5′-GGC ACG AGT TAC AGT TAT GG-3' (Deardorff et al., 1998), *xBtg-x*: F5′-GCA TCT TCC ATA GGC GAC AT3′, R 5′-TTC TGC TCT GGG CTC TTC AT-3'. Tm used was 60°C. Embryos were processed for *in situ* hybridization according to the protocol outlined in (Sive et al., 2000).

Morpholinos and cDNA constructs

GATA-2 (GATA-2a: 5′-CTT CCA TCG CAG GAG CAA AGT TCT C and GATA-2b: 5′-GGT CAG TAG CCA CTT CCA TTG CAG G-3') (Dalgin et al., 2007) and Wnt11 morpholino antisense oligonucleotides (5'- CCA GTG ACG GGT CGG AGC CAT TGG T-3') (Pandur et al., 2002) were purchased from Gene Tools, LLC (Philomath, OR), along with a standard control MO. DshΔDEP cDNA was generated in the Moon lab (Miller et al., 1999) and purchased from

Addgene (Addgene plasmid 16785). All cDNAs were subcloned into pCS2+ for RNA transcription.

Results

Signals from the ectoderm are required to induce blood formation in the mesoderm prior to stage 13

In order to facilitate identification of GATA-2 targets in the ectoderm that might be required for RBC formation, we first wanted to determine the precise developmental window during which the ectoderm is required for erythropoiesis. To do this, ectoderm was removed from embryos at successive stages of development, from mid-gastrulation (stage 11) and continuing through early neurulation (stage 13). In order to eliminate potential contributions from cells of the anterior VBI, which constitute a distinct lineage of tissue macrophages (Tashiro et al., 2006), the dorsal half of the embryo was removed for these studies. Thus, only the ventral half of the embryo, which gives rise to the majority of cells in the VBI, was used for subsequent analysis. Following isolation of the ventral half of the embryo, the ectoderm was either retained (Fig. 3-1A, C) or removed (Fig. 3-1B, D, E) at the stages indicated, and the remaining segments were cultured until wild type siblings reached the tailbud stage (stage 34). The dissected embryos were then assayed by double label *in situ* hybridization for expression of *Xpox2* (Fig. 3-1, purple stain) and *globin* (turquoise stain), markers of white and red blood cell differentiation, respectively. Expression of *Xpox2* was absent in embryos in which the ectoderm was removed at stage 11 (Fig. 3-1C),

but present when ectoderm was removed after stage 12 (Fig. 3-1D, not shown). Thus, ectodermal factors are required for differentiation of the subset of circulating myeloid cells derived from the ventral side of the embryo, and these

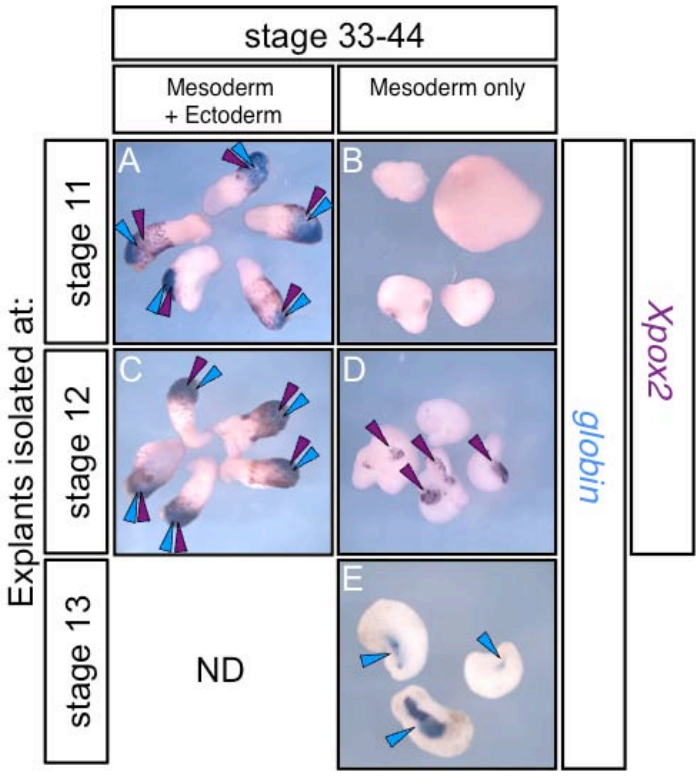


Figure 3-1. Signals from the ectoderm are required to induce blood formation in the mesoderm during gastrulation. The ectoderm was either retained (A, C) or removed (B, D, E) from embryos at stages 11 (A, B), 12 (C, D) or 13 (E). To eliminate contributions from the anterior blood island, the dorsal half of all embryos was also removed when explants were isolated. Embryos were allowed to develop until intact siblings reached the tailbud (stage 33-4) and assayed for expression of *globin* (A-E, turquoise stain, blue arrowheads) and *Xpox2* (A-D, purple stain, purple arrowheads) by *in situ* hybridization. ND = not done.

signals have been transmitted by stage 12. By comparison, *globin* expression was absent in embryos in which ectoderm was removed at stages 11 and 12 (Fig. 3-1B, D), but present when ectoderm was removed at stage 13 (Fig. 3-1E). These results provide the first evidence that non-cell autonomous signals from the ectoderm are required during primitive myeloid development. In addition, we have precisely defined the stages of development during which blood formation from the ventral mesoderm transitions to becoming independent of ectodermal signals for both white and red blood cells. Specifically, we find that by stage 12, the ventral mesoderm is competent to form white blood cells (WBCs) but not

RBCs in the absence of ectoderm. However, by stage 13, the requisite ectodermal signals have been transmitted and the mesoderm is competent to form both WBCs and RBCs independently.

A microarray approach to identify GATA-2 targets in the ectoderm that are required to induce blood formation in the mesoderm

Although ectodermal GATA-2 is essential for erythropoiesis (Dalgin et al., 2007), it is a transcription factor and thus unable to itself mediate the signal which must be transmitted from ectoderm to hematopoietic mesoderm. We were therefore interested in identifying GATA-2 targets that could mediate a signal between the two germ layers. To identify genes downstream of GATA-2 that are expressed in the ectoderm during the window of development in which ectoderm is required for erythropoiesis, we used microarray analysis to compare gene expression profiles from wild type and GATA-2 depleted ectoderm. We therefore dissected ectodermal explants just prior to gastrulation (stage 9-10), and cultured them to the end of gastrulation (stage 12). The ectodermal explants were then harvested and RNA was isolated for hybridization to a microarray (Illustrated in Fig. 3-2).

Because *Xenopus laevis* is not an isogenic model system, we anticipated a high degree of background variability. We therefore used two additional conditions to help us navigate our array data and to increase the specificity of our list of candidate genes. First, we analyzed gene expression in ectoderm isolated

from *Friend of GATA* (*FOG*) injected embryos. We have shown that overexpression of FOG in the mesoderm dominantly interferes with GATA function, causing disruption of erythropoiesis (Chapter 2). To verify that this

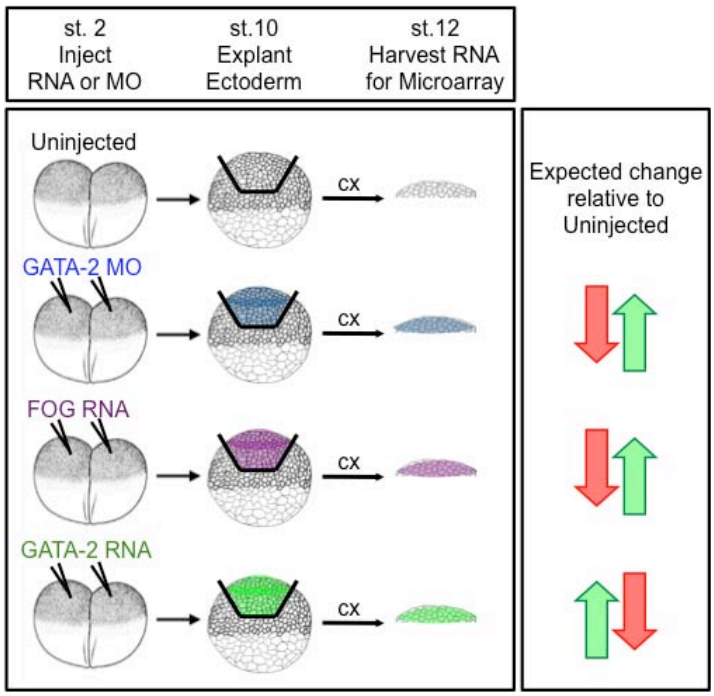
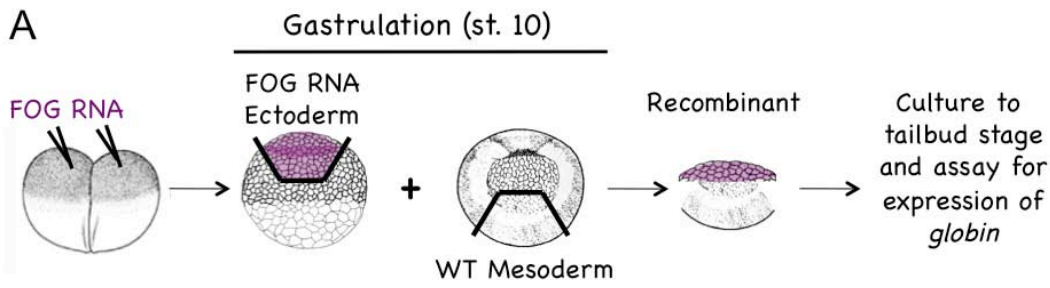


Figure 3-2. Microarray approach to identify ectodermal GATA-2 targets required for blood formation in the mesoderm. Schematic of microarray strategy and sample acquisition. Embryos were injected at the two-cell stage with GATA-2 MO, FOG RNA or GATA-2 RNA and cultured to the early gastrula stage (stage 10). At stage 10, ectoderm was explanted and cultured to stage 12, at which point ectodermal explants in each group were pooled and RNA was extracted for microarray analysis.

dominant-negative relationship also applies to GATA-2 function in the ectoderm, we showed that ectoderm isolated from embryos injected with FOG RNA was unable to support erythropoiesis when recombined with ventral mesoderm (Supp. Fig. 3-S1). Thus, changes in gene expression in FOG overexpressing ectoderm should mirror those observed in GATA-2 morphant ectoderm (illustrated in Fig. 3-2). Second, we analyzed ectoderm isolated from embryos injected with GATA-2 mRNA. If overexpressed GATA-2 is sufficient to induce target gene expression, then the subset of genes that show parallel changes in expression upon GATA-2 MO or FOG RNA injection, would show changes in expression in the opposite direction upon overexpression of GATA-2 (Fig. 3-2).



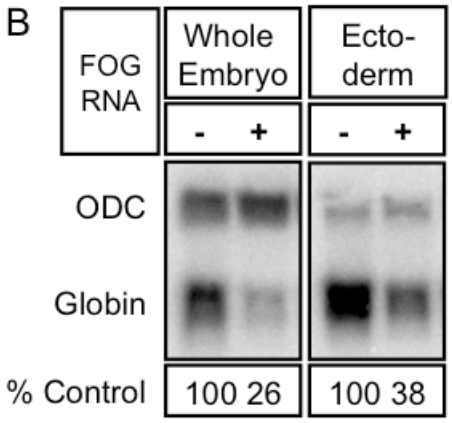


Figure 3-S1. Overexpression of FOG in the ectoderm blocks blood formation when recombined with wild type mesoderm. (A) Schematic of ectoderm-mesoderm recombination assay. (B) Embryos were injected into both cells at the two-cells stage with FOG RNA. At stage 10, ectoderm was explanted from uninjected controls or from embryos injected with FOG RNA. Ectodermal explants were co-cultured with ventral mesoderm explanted from wild type embryos until intact siblings reached the tailbud stage and analyzed for expression of *globin* by Northern blotting. Levels of *globin* are normalized to *ODC* and reported as a percentage of control below each lane.

Together, these conditions provided us with two additional ways to validate our GATA-2 morphant dataset. We used these predicted relationships to execute a preliminary screen for targets that would be good candidates for playing a role in regulating erythropoiesis (*i.e.*, genes that showed parallel changes in expression in GATA-2 MO and FOG RNA injected ectoderm, and opposite changes in GATA-2 RNA injected ectoderm). Using these criteria, we identified 750 genes that showed a greater than 1.2-fold change and 150 genes that showed a greater than two-fold change in the predicted directions for at least two conditions. A list of candidate genes most relevant to this study, along with the fold-change relative to uninjected controls, is presented in Table 3-1.

GENE	PATHWAY/ ASSOCIATION	FOLD CHANGE vs. UNINJECTED		
		GATA-2 MO	FOG RNA	GATA-2 RNA
Wnt-11	Non-canonical Wnt	-3.87	-2.9	2.2
cdc42 GEF	Non-canonical Wnt	-2.02	-1.8	2.4
Ephrin-B2b	Non-canonical Wnt	-1.91	-2.2	1.9
Frizzled 7	Non-canonical Wnt	-1.47	-1.6	1.4
Frizzled 8	Canonical Wnt	1.9	1.9	1.1
c-Jun	Canonical Wnt target & Stem/Progenitor Cell Mkr	3	3.5	-1.4
Btg-x	Canonical Wnt target	2.0	2.5	-5.6
Id2	Canonical Wnt target & Stem/Progenitor Cell Mkr	1.9	1.9	1.1
c-myb	Stem/Progenitor Cell Mkr	5.1	5.4	-2.4
Sca2	Stem/Progenitor Cell Mkr	4.2	9.9	1.0
Hesx	Stem/Progenitor Cell Mkr	3.5	2.9	-3.0

Table 3-1. Genes identified by the microarray analysis downstream of GATA-2 that are predicted to be relevant for hematopoiesis. *Btg-x=B-cell translocation gene x*; Id2=*Inhibitor of DNA binding 2*; *Sca2=Stem cell antigen 2*

Our microarray data yielded several interesting trends, which were used to guide our initial analysis. First, genes associated with the non-canonical and canonical Wnt pathways appeared to be reciprocally regulated downstream of GATA-2, such that genes in the non-canonical Wnt pathway were down regulated in GATA-2 morphant ectoderm, but were positively regulated by GATA-2, whereas members of the canonical Wnt pathway and their downstream effectors were up regulated in GATA-2 morphants, and were negatively regulated by GATA-2 (Table 3-1). Interestingly, microarray target genes associated with the non-canonical Wnt/PCP pathway were predominantly in the class of pathway components. For example, Wnt11 is a ligand that can signal through the receptor

Fz7, EphrinB2b can activate the PCP pathway through interaction with Disheveled (Lee et al., 2006; Tanaka et al., 2003; Wang, 2009), and cdc42GEF activates the small GTPase cdc42, a downstream effector of both Wnt/PCP and Wnt/Ca^{2+} signaling (Schlessinger et al., 2009). Regulation of non-canonical Wnt pathway components by GATA-2 suggests that activation of this pathway may be direct. In contrast, genes identified by our microarray analysis associated with the canonical pathway are primarily canonical Wnt transcriptional targets, consistent with this pathway being activated by other mechanisms and then being indirectly repressed by GATA-2.

A second trend predicted by our microarray analysis was that genes associated with proliferation and hematopoietic stem cell maintenance, such as *c-myb* and *Stem cell antigen 2* (Sca2), were upregulated in GATA-2 morphant ectoderm. Both of these genes must be downregulated to promote exit from the progenitor state and allow for differentiation during definitive hematopoiesis (Bresson-Mazet et al., 2008; Yanagisawa et al., 1991). Induction of markers of the progenitor state thus suggests a potential defect in the ectoderm's ability to properly differentiate in the absence of GATA-2 (Table 3-1). This in turn could prevent development of signals required for blood formation in the mesoderm.

Non-canonical Wnt pathway members are upregulated and canonical Wnt pathway members are downregulated downstream of GATA-2 in the ectoderm

To validate changes in gene expression predicted by the microarray analysis, we performed qPCR on ectodermal explants (Fig. 3-3A) isolated from embryos that had or had not been injected with GATA-2 MO or FOG RNA. Using qRT-PCR, we tested a subset of genes identified by the microarray from both the non-canonical (x*Wnt11, EphrinB2, cdc42 GEF*) and canonical Wnt (*xFz8*, *x-Btgx*) pathways (Fig. 3-3A). In preliminary studies, we find that the trends predicted by the microarray were validated, such that genes associated with non-canonical Wnt signaling were strongly repressed in both the GATA-2 MO and FOG RNA injected ectodermal explants, whereas those associated with the canonical pathway were activated in response to GATA-2 MO or FOG RNA injection.

As an alternate approach to validating these relationships, we repeated the injections described above and performed *in situ* hybridization on whole embryos at stage 13-14 to determine whether these genes were expressed in ectodermal cells and if the changes in expression of microarray targets were also evident in intact embryos (Fig. 3-3B-G). By injecting only one cell at the two-cell stage, these studies also allowed us to determine if the resultant changes in expression are mediated by a cell-autonomous mechanism. As shown in Fig. 3-3 B-G, cdc42GEF was expressed in ventrolateral ectoderm during the gastrula stage (purple arrowheads). It is thus expressed at the right time and place to be a GATA-2 target. Consistent with the microarray and qPCR analysis, expression of this gene was unilaterally reduced in the ventral ectoderm upon unilateral overexpression of FOG RNA (yellow arrowheads; Fig. 3-3B-D compared with Fig. 3-3E-G). For additional verification, studies with the full complement of

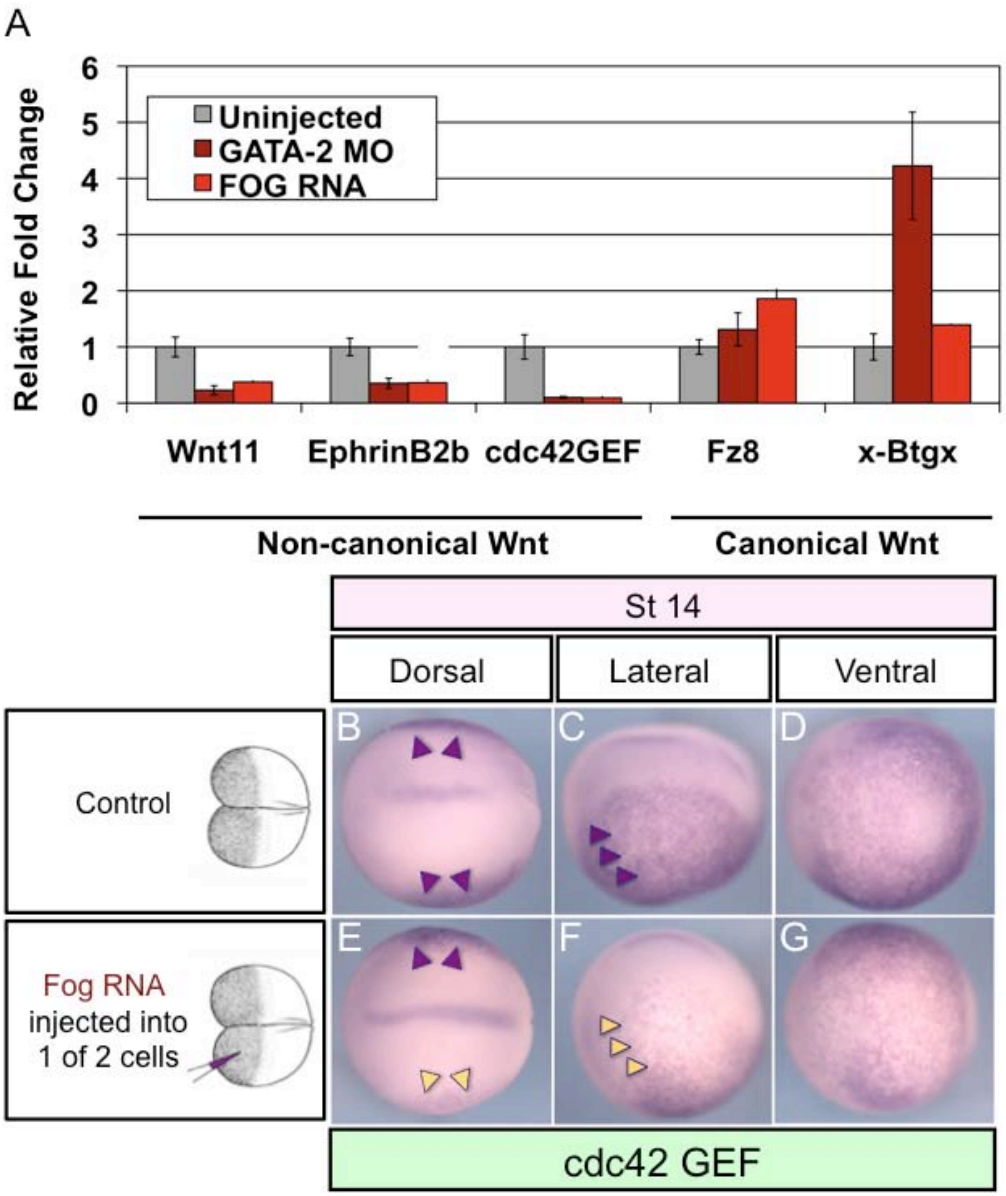


Figure 3-3. Non-canonical and canonical Wnt pathway members are reciprocally regulated downstream of GATA-2 in the ectoderm. (A) Both blastomeres of embryos at the two-cell stage were injected with either GATA-2 MO or FOG RNA. Ectoderm was harvested at stage 10, cultured to stage 12 and RNA harvested for qPCR analysis to validate changes in expression of non-canonical (*Wnt11, Ephrin B2, cdc42 GEF*) or canonical Wnt (*Fz8, x-Btgx*) pathway members as predicted by the microarray analysis. (B-G) Control embryos (B-D) or embryos injected with FOG RNA in one of two cells at the two-cell stage (E-G), were allowed to develop to stage 14 and assessed for changes in *cdc42 GEF* expression by *in situ* hybridization. Anterior is to the right in all cases. Purple arrowheads indicate normal levels of staining. Yellow arrowheads indicate areas of reduced staining.

injection conditions used for the microarray will be performed for other non-canonical and canonical targets identified. In addition, embryos will be examined histologically to determine whether changes in target gene expression are confined to ectodermal cells or are observed in ventral mesoderm as well. Finally, we are currently testing additional genes not identified by the microarray, but that are associated with each pathway, as a means of independently validating the ability of our manipulations to perturb signaling in these pathways. For example, previous studies indicate that the respective activation or repression of the canonical Wnt target genes, *Axin2* or *Lef1,* are reliable readouts of canonical activity (Chien et al., 2009). We will therefore use qPCR to verify that these genes are also changed as predicted downstream of GATA-2.

The finding that non-canonical Wnt pathway components are upregulated and canonical Wnt pathway target genes are downregulated by GATA-2 is interesting given that these two arms of the Wnt pathway have been shown to be alternately required for different phases of development in a number of contexts, including during hematopoiesis (Sinenko et al., 2009; Vijayaragavan et al., 2009). Specifically, canonical Wnt pathway activation is often required to promote stem or progenitor cell proliferation in order to build up a large enough precursor pool to populate an organ (Luis et al., 2009). Subsequent activation of the non-canonical Wnt pathway is then required to inhibit the canonical pathway, thereby ceasing proliferation and enabling cells to become specified or differentiate with a given fate (Vijayaragavan et al., 2009). Our microarray data thus support the hypothesis that GATA-2 activates non-canonical Wnt signaling, which is in turn

required to repress canonical Wnt signaling in order to halt progenitor proliferation and allow mesodermal cells to be specified with a hematopoietic fate during gastrulation.

Markers of proliferating stem and progenitor cells are upregulated in the absence of GATA-2 function

Consistent with the hypothesis that ectodermal GATA-2 is required to dampen canonical Wnt pathway activation in order to enable progenitors to cease proliferating and become specified, our microarray analysis identified markers of stem or progenitor cells as being strongly upregulated in GATA-2 morphant ectoderm (Table 3-1). Given that these genes are known to be required within hematopoietic progenitors, we wished to examine their potential regulation by GATA-2 more closely. We therefore analyzed expression of two such progenitor cell markers, *Sca2* and *c-myb*. c-myb is a transcription factor required during hematopoietic progenitor development, but which must be downregulated in order for blood cells to mature. Forced expression or loss of function of c-myb in erythroid progenitors blocks or induces differentiation, respectively (Clarke et al., 1988; Garcia-Morales et al., 2009), suggesting that this gene functions in RBC progenitor maintenance. The pattern of c-myb regulation identified by our microarray analysis suggests that it may be regulated in a cell autonomous fashion by ectodermal GATA-2. To begin to test this hypothesis, we performed *in situ* hybridization on stage 14 wild type embryos or embryos that had been injected with GATA-2 RNA or FOG RNA (Fig. 3-4 A-I).

We find that *c-myb* is expressed in ventrolateral ectoderm throughout and immediately after gastrulation (Fig. 3-4A-C purple arrowheads, and not shown). Consistent with our microarray analysis, *c-myb* was strongly inhibited by injection of GATA-2 RNA (Fig. 3-4D-F yellow arrowheads), whereas blocking GATA-2 function by injection of FOG RNA resulted in increased *c-myb* expression (Fig. 3-4G-I black arrowheads). Induction of progenitor cell markers such as *c-myb* and *Sca2* upon GATA-2 disruption, is consistent with the hypothesis that GATA-2 normally activates non-canonical Wnt signaling, which in turn is required to keep canonical Wnt signals that prevent differentiation in check. Because we are only able to analyze ectodermal expression by this assay, further analysis of these

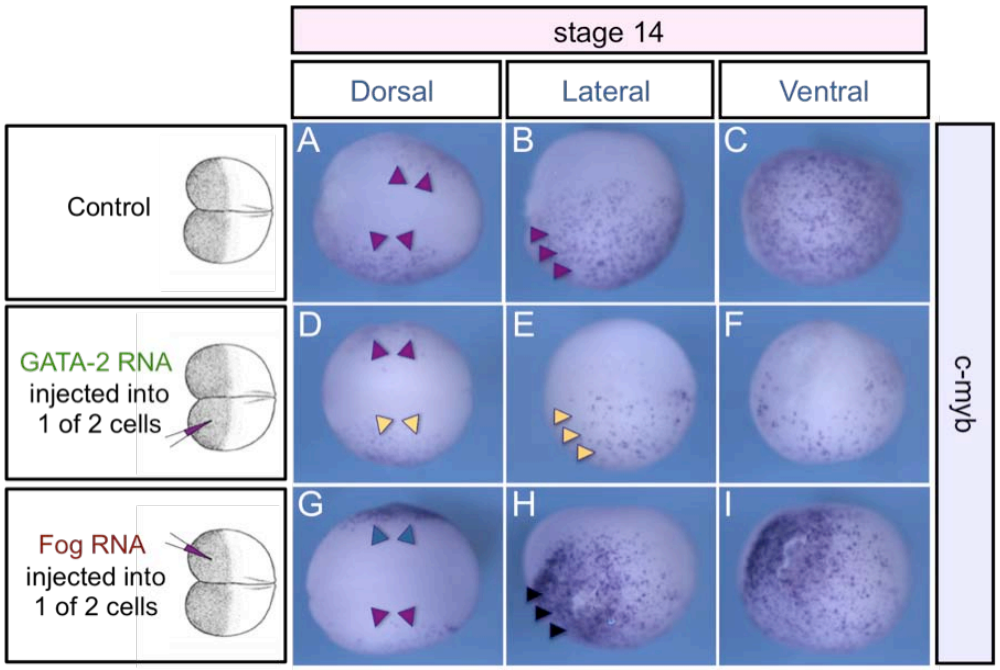


Figure 3-4. Markers of proliferating stem and progenitor cells are upregulated in the absence of GATA-2 function. (A-I) Control embryos (A-C) or embryos injected into one of two cells at the two-cell stage with GATA-2 RNA (D-F) or FOG RNA (G-I), were allowed to develop to stage 14 and assessed for changes in *c-myb* expression by *in situ* hybridization. Purple arrowheads indicate normal levels of staining, yellow arrowheads indicate reduced staining and black arrowheads indicate increased staining. Anterior is to the right in all cases.

embryos by section *in situ* or by qPCR analysis of isolated mesoderm will be necessary to determine whether expression of these genes is also regulated similarly by GATA-2 within hematopoietic mesoderm. This study provides preliminary evidence that disruption of GATA-2 may result in erythropoietic defects by perturbing the balance between progenitor maintenance and differentiation.

Non-canonical Wnt signaling through Wnt11 is required for primitive erythropoiesis

If our prediction is correct, that GATA-2 is required to activate the non-canonical Wnt pathway to enable progenitors to commit to a hematopoietic fate, then inhibition of non-canonical Wnt signaling should lead to loss of blood. To begin to test this hypothesis, we made use of a dominant-negative version of the Wnt effector Disheveled (DshΔDEP), which selectively inhibits non-canonical Wnt signaling (Boutros et al., 1998). Because the DEP domain has been shown to be dispensable for canonical Wnt signaling, β-catenin-mediated signals remain intact in the presence of this construct. This approach is particularly useful as it allowed us to determine whether signaling through the non-canonical Wnt pathway is generally required for primitive erythropoiesis without prior knowledge of the specific ligand involved. We therefore injected DshΔDEP into the animal pole (AP) or into the ventral marginal zone (VMZ) at the four-cell stage to preferentially inhibit non-canonical signaling in the ectoderm or the ventral mesoderm, respectively (Fig. 3-5A). These embryos were allowed to develop to

the tailbud stage (st. 34) and assessed for expression of *globin* by Northern blotting. Interestingly, expression of *globin* was strongly repressed in both the AP and VMZ injected embryos (0% and 4% of controls, respectively), suggesting that non-canonical Wnt signaling may be required in both tissues for primitive erythropoiesis. Although injected RNAs are prevented by their large size from significant migration to other sites within the embryo, additional studies using ectoderm-mesoderm recombinants will be performed to more stringently test for tissue specific regulation by non-canonical Wnts.

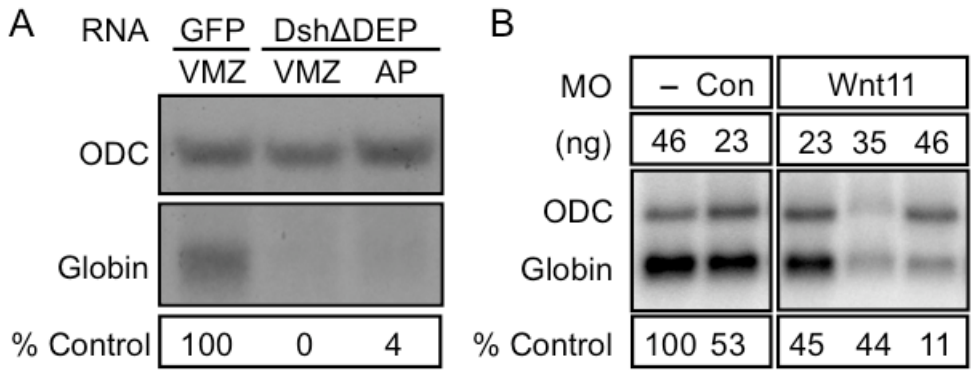


Figure 3-5. Non-canonical Wnt signaling is required for primitive erythropoiesis. (A) GFP control RNA was injected into the ventral marginal zone (VMZ) and DshΔDEP RNA was injected into the VMZ or animal pole (AP) of embryos at the four-cell stage, and expression of *globin* was analyzed by Northern blotting at stage 34. (B) Control MO (Con) or Wnt11 MO were injected into the animal pole of both cells at the two-cell stage and expression of *globin* was analyzed by Northern blotting at stage 34. Levels of *globin* transcripts are normalized relative to expression of *ODC* and reported as a percentage of control below each lane.

To begin to determine which specific ligand might be responsible for initiation of signaling through the non-canonical Wnt pathway during hematopoiesis, we returned to our microarray analysis for clues. Wnt11 was the most obvious candidate as it was positively regulated by GATA-2 and expressed in ectoderm at stage 12. While it is known to play a key role in cardiac

development (Flaherty and Dawn, 2008), a potential role in blood formation has not yet been tested. In addition, because Wnt11 is a secreted protein, it is a good candidate to mediate the signal between ectoderm and mesoderm required for blood. To begin to address this hypothesis, we injected an antisense Wnt11 MO that has been shown to specifically block translation of Wnt11 mRNA (Pandur et al., 2002) into both animal pole cells of embryos at the two-cell stage. These embryos were allowed to develop to the tailbud stage and assayed for expression of *globin* by Northern blotting. As shown in Fig. 3-5B, injection of Wnt11 MOs led to a dose dependent reduction in expression of *globin*. These experiments will be repeated along with appropriate controls, such as rescuing the morphant phenotype with Wnt11 mRNA that lacks the MO binding site, to demonstrate specificity. Taken together, these studies are consistent with ectodermal GATA-2 being required to activate non-canonical Wnt signaling, possibly via Wnt11, in order to promote primitive RBC development.

Hyperactivation of the canonical Wnt pathway during gastrulation represses primitive erythropoiesis

Based on the above data, our working hypothesis is that activation of non-canonical Wnt signaling downstream of GATA-2 during gastrulation represses canonical Wnt signaling. Dampening of canonical Wnt signals is in turn required to allow progenitors to cease proliferation and become specified as erythrocytes. If this hypothesis is correct, then aberrant activation of the canonical Wnt pathway during this same time period would repress hematopoiesis. To test this

prediction, we treated gastrula-stage embryos with LiCl, a potent inhibitor of GSK3β, which is a member of the β-catenin destruction complex. LiCl treatment thus results in stabilization of β-catenin and increased signaling through the canonical Wnt pathway (Sive et al., 2000). This system is particularly useful in that LiCl can be easily removed by several brief washes in culture medium, permitting temporally specific activation of the canonical pathway.

To determine if inappropriate canonical Wnt signaling during gastrulation inhibits RBC development, embryos were treated at stage 10 with a 20 minute pulse of LiCl, allowed to develop to stage 18 or 34, and assessed for changes in expression of the specification and differentiation markers, *Stem cell leukemia* (*Scl*), and *globin* (stage 34 only), respectively (schematized in Fig. 3-6A). Aberrant activation of canonical Wnt signaling at the onset of gastrulation lead to a dramatic reduction in expression of *Scl* at both stages 18 and 34, indicating that specification of hematopoietic fate has been perturbed (Fig. 3-6B). Similarly, expression of *globin* was also reduced at stage 34 (Fig. 3-6B) in these embryos. Together, these data demonstrate that hematopoietic fate is not properly specified when canonical Wnt signaling is upregulated during gastrulation, which likely results in subsequent failure of RBCs to differentiate. We next wished to determine the developmental time-frame during which upregulation of canonical Wnt signaling could inhibit erythropoiesis. We therefore treated embryos with a single 20 minute pulse of LiCl at stages that encompass gastrulation, stages 9, 10, 11, 12, or 13. These embryos were then allowed to develop to stage 34 and changes in *globin* expression were assessed by Northern blotting (Fig. 3-6C). We

found that transient activation of canonical Wnt signaling at stages 9, 10, or 11 was sufficient to repress RBC development. When treated with LiCl at stage 12, expression of *globin* was less strongly repressed and by stage 13, was no longer sensitive to LiCl.

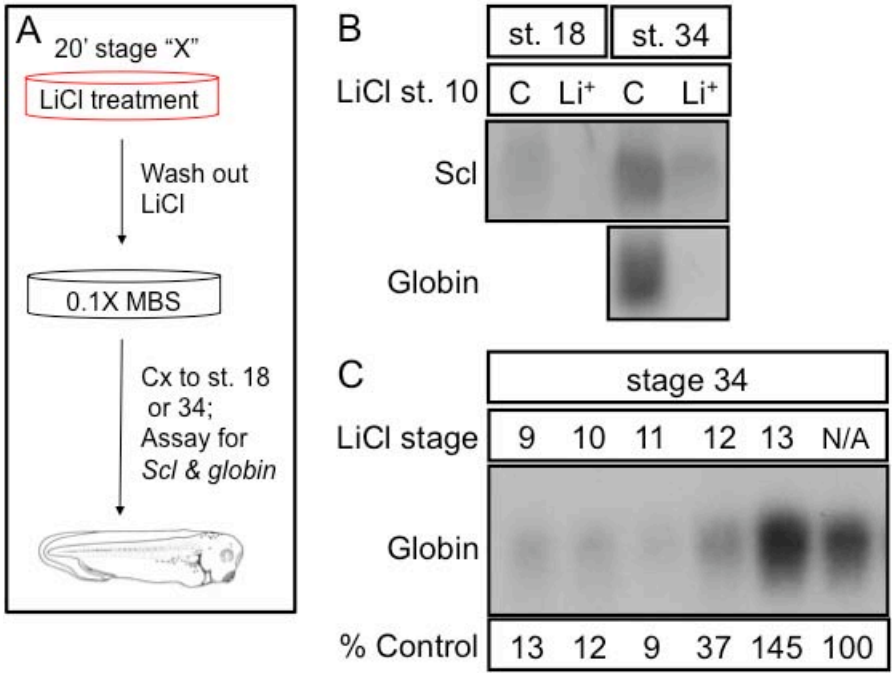


Figure 3-6. Hyperactivation of the canonical Wnt pathway during gastrulation represses blood development. (A) Schematic of approach used to transiently activate canonical Wnt signaling. Briefly, embryos at the stages specified were incubated in LiCl solution for 20 minutes, followed by several washes in 0.1X MBS culture medium. Embryos were allowed to develop to stage 18 or 34 and assayed for expression of *globin* and/or *Scl*. (B) Embryos were treated with LiCl as described in (A) at stage 10, allowed to develop to stage 18 or 34 and assayed for expression of *Scl* and *globin* (st. 34 only) by Northern blotting. (C) Embryos were treated with LiCl as described in (A) at stages 9, 10, 11, 12 or 13, allowed to develop to stage 34 and expression of *globin* was determined by Northern blotting. Levels of *globin* transcripts are normalized and reported as a percentage of *globin* levels in untreated embryos below each lane.

Interestingly, the window of development during which hyperactivation of canonical Wnt signals represses erythropoiesis precisely coincides with that

during which ectodermal signals are required (Fig. 3-1). Together, these observations are consistent with our hypothesis that GATA-2 mediated activation of non-canonical Wnt signaling in the ectoderm during gastrulation is required to dampen canonical Wnt signals for normal commitment of mesoderm to an erythroid fate.

Discussion

Reciprocal regulation of non-canonical and canonical Wnt signaling downstream of GATA-2 in the ectoderm is required for primitive erythropoiesis

Previous studies in our lab have determined that the transcription factor GATA-2 functions in the ectoderm to generate signals required for erythropoiesis in the mesoderm. In the current study, we have used microarray analysis as an unbiased method to identify GATA-2 transcriptional targets that are required for primitive erythropoiesis. Using this approach, we found evidence that non-canonical Wnt signaling may be activated and canonical Wnt signaling repressed downstream of GATA-2. In addition, we found that genes required for progenitor maintenance were downregulated by GATA-2. Together, these observations led us to the hypothesis that ectodermal GATA-2 is required to activate non-canonical Wnt signaling, which in turn represses canonical Wnt target genes. Repression of canonical Wnt targets is necessary to permit exit from the progenitor state and allow for subsequent RBC specification, and thus differentiation (see Fig. 3-8 for a model). While this notion of a biphasic

requirement for canonical and non-canonical signals has been previously suggested based on hematopoietic differentiation assays in embryonic stem (ES)

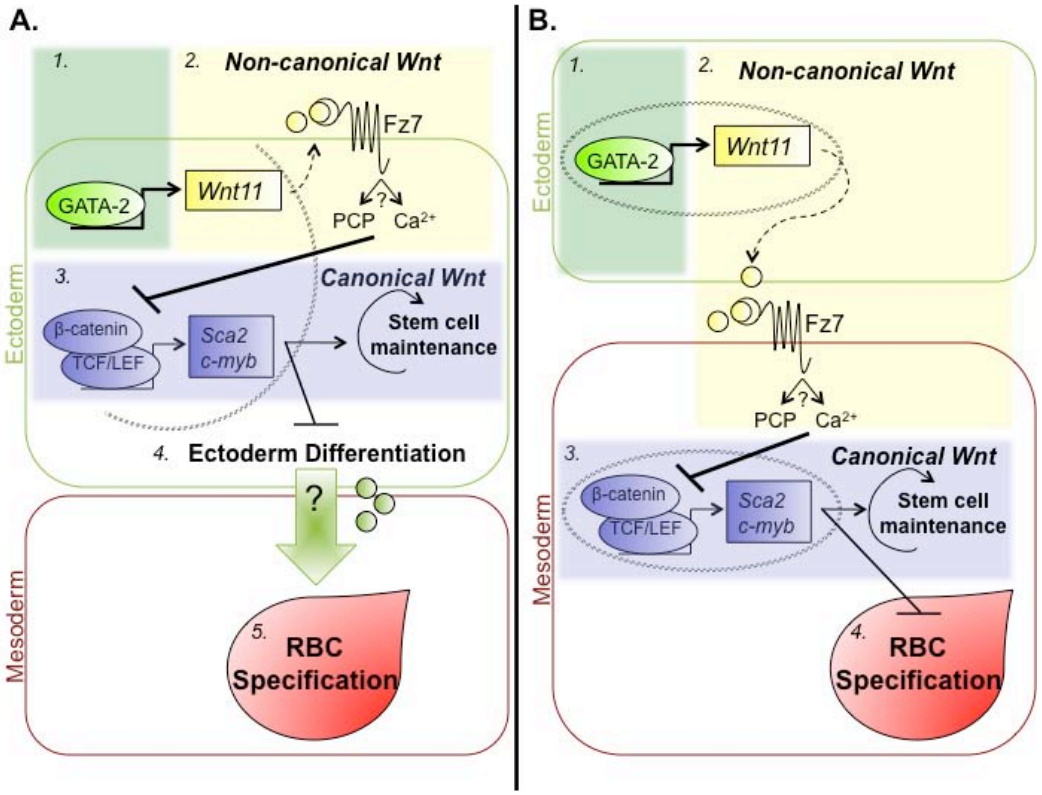


Figure 3-8. Models for potential mechanisms of regulation of primitive erythropoiesis by GATA-2 and canonical and non-canonical Wnt signaling. (A) GATA-2 activates non-canonical Wnts, which repress canonical Wnts, allowing for ectoderm differentiation. This in turn permits the development and transmission of signals that support RBC specification in the mesoderm. (B) GATA-2 activates non-canonical Wnts in the ectoderm, which signal to the mesoderm to repress hematopoietic progenitor maintenance by canonical Wnts and promotes RBC specification.

cell models (Vijayaragavan et al., 2009), our study is the first to provide evidence of such a requirement *in vivo*.

Non-canonical and canonical Wnt signaling play distinct and opposing roles during development

The literature is replete with studies that demonstrate that the canonical Wnt pathway activates proliferative signals in many contexts, including during epidermal, intestinal, and mammary development (Chien et al., 2009). Moreover, failure to properly regulate these signals is the source of many human cancers and the leading cause of gastrointestinal tumors (Chien et al., 2009; Polakis, 2007). Our studies are consistent with a model in which canonical Wnt signaling is responsible for induction and maintenance of genes like *Sca2* and *c-myb*. Thesis genes are known to promote progenitor self-renewal, during erythropoiesis and may have a similar function during ectoderm development. We have also provided evidence that GATA-2 induced non-canonical Wnt signaling is an important regulatory mechanism for keeping canonical signals in check. These observations may be useful in the future for development of therapies for malignancies in which hyperactive canonical Wnt signaling is the primary defect. In subsequent studies, it will be important to determine the precise molecular mechanism by which non-canonical Wnt signaling regulates the canonical pathway. Several studies have shown that non-canonical signals can directly repress canonical targets, however the mechanisms by which they do so appear to be context dependent (Mikels and Nusse, 2006; Topol et al., 2003; Weidinger and Moon, 2003). In order to begin to determine how this cross-regulation is achieved during primitive erythropoiesis, we must first identify which non-canonical pathway effectors are activated and in which tissue they function.

The Wnt/PCP and the Wnt/Ca^{2+} pathways are two potential downstream mediators of non-canonical Wnt signaling during primitive erythropoiesis

The number of distinct signaling molecules identified downstream of non-canonical Wnts is ever expanding and includes, Ca^{2+}/Calmodulin Kinase family members, JNK, small GTPases Rho and Rac, and Nuclear Factor of Activated T cells (NFAT) (Boutros et al., 1998; Chien et al., 2009; Dejmek et al., 2006; Habas et al., 2003; Kuhl et al., 2000; Sheldahl et al., 2003). Two pathways that have been previously implicated in hematopoietic development are the Wnt/Ca^{2+} pathway and the Wnt/PCP pathway, which signals through activation of JNK (Boutros et al., 1998; Yamanaka et al., 2002). JNK signaling is a particularly attractive candidate given that this pathway is directly activated downstream of Wnt11 during cardiac development (Afouda et al., 2008; Pandur et al., 2002). On the other hand, the Wnt/Ca^{2+} pathway has been shown to be essential during multiple steps of hematopoietic development in cell culture models (Vijayaragavan et al., 2009). Our initial studies are thus aimed at determining whether either of these two pathways is activated or repressed upon overexpression or loss of ectodermal GATA-2, respectively.

Non-canonical Wnt signaling in the ectoderm may inhibit canonical Wnt targets in the mesoderm to promote normal differentiation of primitive erythrocytes

Initial experiments to determine whether non-canonical Wnt signaling is required in the ectoderm, the mesoderm or in both tissues will necessitate using

tissue recombinants (schematized in Fig. 3-S1) to knock down non-canonical Wnt signals in the specific germ layers and assaying for alterations in expression of RBC specification and differentiation markers. Because the present study has identified Wnt11 as a non-canonical Wnt ligand required for primitive erythropoiesis downstream of GATA-2, we are currently using MOs to deplete Wnt11 in the ectoderm to test whether it is specifically required in this tissue for blood. To more definitively ascertain if Wnt11 must itself be transmitted from ectoderm to mesoderm, or, alternatively, if it induces a secondary signal, we will also ask whether loss of non-canonical Wnt signaling in the mesoderm also disrupts erythropoiesis. Because Wnt11 is a soluble factor, generating recombinants with wild type ectoderm could confound analysis of Wnt11 depleted mesoderm. We will therefore use the dominant-negative Disheveled construct, DshΔDEP, to selectively disrupt intracellular transduction of non-canonical signals in either the ectoderm or the mesoderm. These experiments will test the hypothesis that Wnt11 is required specifically in the ectoderm for primitive erythropoiesis and will determine whether non-canonical signaling is also required in the mesoderm to induce primitive erythropoiesis.

If non-canonical Wnts are in fact required for specification of hematopoietic fate in the mesoderm, our above data would suggest that one potential mechanism by which they do so is through repression of proliferative signals mediated by canonical Wnts. To more stringently test this hypothesis, we would need to verify that canonical signals must be dampened specifically in the mesoderm. We have therefore initiated studies in which LiCl has been used to

selectively hyperactivate canonical Wnt signaling in either the ectoderm or the mesoderm during gastrulation. In preliminary experiments, we find that hyperactivation of canonical Wnt signaling in the mesoderm represses blood when recombined with wild type ectoderm in 3/3 studies. However, the reciprocal experiment in which only ectoderm has been treated with LiCl has no effect on expression of *globin* in 2/3 studies, supporting our hypothesis that the requisite downregulation of canonical Wnt signals during gastrulation occurs in mesodermal cells.

Although our model predicts that markers of progenitor cells are negatively regulated by canonical signals downstream of GATA-2, we have not directly demonstrated this relationship. Additional studies will thus be necessary to show that expression of genes such as *c-myb* and *Sca2* are in fact altered upon perturbation of canonical Wnt signaling. In support of our hypothesis that stem cell maintenance genes are regulated by canonical signals, other genes identified in our microarray analysis as repressed downstream of GATA-2 include *c-jun* and *Inhibitor of DNA binding 2* (*Id2*). Both genes are known to promote proliferation at the expense of differentiation in certain contexts and have been implicated in the pathogenesis of human malignancies (Elagib et al., 2004; Nishimori et al., 2002; Vleugel et al., 2006). Interestingly, Id2 and c-Jun have also been shown to be directly activated by β-catenin (Memezawa et al., 2007; Staal et al., 2004). Based on our above data, we would predict that *Sca2 and c-myb*, which like *Id2 and c-Jun* also function in progenitor maintenance, may be similarly regulated by canonical Wnt signaling.

Our initial objective was to identify a soluble signaling molecule produced in the ectoderm downstream of GATA-2 that is required to support primitive erythropoiesis in the mesoderm. To this end, we identified Wnt11 as a GATA-2 regulated molecule produced in ectoderm during gastrulation that is required for erythropoiesis. However, we have yet to test whether expression of Wnt11 in the mesoderm is sufficient to rescue loss of ectodermal GATA-2. Our current efforts are focused on addressing this very interesting question.

GATA transcription factors mediate the switch between canonical and non-canonical Wnt signaling during development

Our microarray analysis and subsequent embryological studies suggest a model in which GATA-2 regulates the balance between non-canonical and canonical Wnt signaling. This critical equilibrium between progenitor maintenance and differentiation is an important topic of study given the potential oncogenic consequences of dysregulation. Our data suggesting that GATA-2 plays a role in maintaining the balance between canonical and non-canonical Wnt signaling is consistent with previous findings in both heart and lung development. In these tissues, GATA-4 and -5, or GATA-6, are similarly required to regulate non-canonical and canonical Wnt signals in order to achieve a normal balance between proliferation and differentiation during development. For example, in the lung epithelium, GATA-6 directly activates the non-canonical Wnt receptor, Frizzled 2 (Fz2), which in turn represses canonical Wnt signaling. Hyperactivation of the canonical pathway due loss of GATA-6 or by ectopic

expression of constitutively active β-catenin, results in excessive progenitor proliferation at the expense of differentiation in the pulmonary epithelium, during both embryonic development and regeneration following injury in adults (Zhang et al., 2008). Separate studies on cardiac development have shown that GATA-4 is required to activate Wnt11 and that induction of non-canonical Wnts by GATA-4 and -6 is required to repress canonical Wnts for normal differentiation (Afouda et al. 2008). Together, these studies have many parallels to the signaling pathway we have uncovered during primitive erythropoiesis, and suggest that this may be a general mechanism for regulating embryonic development, tumor suppression, and possibly for regulating response to injury in adults, as described for the lung.

Evidence for biphasic regulation of hematopoiesis by canonical and non-canonical Wnt pathways

Studies of *Drosophila* hematopoiesis have demonstrated a dual and biphasic role for *Wg* signaling during hemocyte development, both within hemocytes as well as in the surrounding niche cells (Sinenko et al., 2009). In flies, Wg signaling via the canonical Wnt pathway is required for proliferation of both hematopoietic progenitors and the surrounding niche cells during distinct stages of hemocyte maturation. Moreover, these Wg mediated proliferative signals are also required during multiple stages of hemocyte development (Sinenko et al., 2009). Our microarray data together with *in situ* data in whole embryos demonstrate that genes that promote hematopoietic progenitor

proliferation, such as c-myb and Sca2, are regulated by GATA-2 in the niche-like ectodermal cells. The observation that c-myb, Sca2 and canonical Wnts are similarly derepressed upon loss of GATA-2, suggests the possibility that similar to in flies, canonical Wnt signaling is required both in the ectodermal niche cells and in mesodermal precursors to regulate proliferative phases of hematopoiesis in vertebrates. Alternately, GATA-2 may simply be required to promote normal differentiation of ectoderm, and in doing so downregulates genes such as c-myb and Sca2, which are active during progenitor maintenance. Properly differentiated ectoderm would in turn produce signals required to induce erythropoiesis in the mesoderm. Additional studies are necessary to determine whether c-myb and Sca2 are acting in the ectoderm, mesoderm or both and whether activation or repression in either of these tissues alters RBC development.

Canonical and non-canonical Wnt signaling are alternately required during distinct phases of development to regulate the balance between proliferation and differentiation

As most previous studies that examine the relationship between canonical and non-canonical Wnts have not addressed how these pathways are regulated over time, it is difficult to interpret published data in a temporally relevant fashion. The current study is unique in that we have begun to examine the effect of manipulating signaling through these pathways in a time-sensitive manner *in vivo*. Though it is clear that canonical Wnt activity is essential during other

phases of hematopoietic development (such as during ventral mesoderm specification and hematopoietic progenitor proliferation (Christian and Moon, 1993; Luis et al., 2009), we have observed that repression of canonical Wnt signals is also required for normal erythroid commitment, but during a very specific window of development. These findings are consistent with a recent study in a human ES cell *in vitro* differentiation model demonstrating that coordinate activation of canonical and non-canonical Wnt signals is required to promote alternating phases of expansion and differentiation respectively (Vijayaragavan et al., 2009). Transitions between these phases corresponded to developmental transitions that occur from stem cells to mesodermal progenitors and from mesodermal progenitors to committed hematopoietic progenitors (Vijayaragavan et al., 2009). Our future studies will focus on testing the hypothesis that a biphasic pattern of regulation by canonical and non-canonical Wnt pathways is required during alternating stages of proliferation and differentiation throughout primitive erythropoiesis, and to ascertain whether these transitions are regulated by GATA transcription factors.

The requirement for signals from non-hematopoietic cells is evolutionarily well conserved

In the mouse embryo, prospective blood forming cells require an extrinsic signal from the adjacent visceral endoderm in order to properly differentiate (Belaoussoff et al., 1998). The primitive, or visceral endoderm is a secretory epithelium, which provides inductive signals to adjacent tissue required for

survival and differentiation (Spyropoulos and Capecchi, 1994). Primitive erythrocytes are derived from mesodermal cells, which arise from the posterior primitive streak and migrate into the extra-embryonic region. During gastrulation, these cells migrate such that they are adjacent to, and thus poised to receive signals from, the visceral endoderm (reviewed by (Baron, 2001). A similar requirement for extrinsic signals is also present during avian hematopoiesis (Miura and Wilt, 1969). The identity of this signal, however, has hitherto remained unknown. In these systems, GATA-2 function has only been examined in blood cells, therefore future studies could address whether it is also expressed and/or required in the adjacent tissues (such as in the VE in mouse), which could serve a hematopoietic niche function during primitive erythropoiesis in higher vertebrates.

Our previous studies have shown that GATA-2 is required in the ectoderm to generate a non-cell autonomous signal required for hematopoiesis in the mesoderm. In the current study, we have used microarray analysis to identify novel roles for non-canonical and canonical Wnt signaling downstream of GATA-2 in primitive erythropoiesis. Our data are consistent with Wnt11 being the ligand for this signal but they do not rule out the possibility that other non-canonical Wnt ligands are also required, or that non-canonical Wnts initiate an unknown secondary signal that is then transmitted to the mesoderm. Further refining this pathway and its regulation will be the subject of future studies described above and in Chapter Five.

CHAPTER 4

Identification of a novel protein, xTRIL, involved in primitive erythropoiesis and non-canonical Wnt signaling

Mizuho S. Mimoto, Sunjong Kwon and Jan L. Christian

Preface

This manuscript is currently in preparation.

- My contributions to this manuscript include microarray target validation by qPCR and *in situ* hybridization, cell movement assays, xTRIL/Wnt epistasis assays (Figs. 4-1A, 4-5G-H, 4-7), and writing the manuscript.
- Sunjong Kwon contributed to this study by performing xTRIL localization and xTRIL overexpression experiments (Figs. 4-3, 4-5I-K).
- Jenelle Johnston contributed to this study by sequencing and generating *in situ* probes for target validation.
- Jan L. Christian contributed to this study by cloning *Xenopus TRIL*, performing *in situ* expression assays, target validation by *in situ* hybridization and Northern blotting, xTRIL MO phenotype analysis, xTRIL overexpression phenotype analysis, Keller explants (Figs. 4-1B-G, 4-2, 4-4, 4-5A-F, 4-6), and by editing the manuscript. She also provided advice and suggestions on experimental design and data analysis, as well as the space, equipment, and supplies with which to perform the experiments.

Abstract

Primitive erythropoiesis, which produces the first red blood cells in the embryo, is regulated in a non cell-autonomous fashion that has been conserved across evolution from frogs to mammals. For example in *Xenopus*, signals from the ectoderm are required to permit the underlying mesoderm to adopt an erythroid fate. Previous work in our lab indicating that the transcription factor GATA-2 is required in ectoderm for erythropoiesis was used as the basis for a microarray analysis in which we compared GATA-2 morphant and wild type ectoderm. Our initial microarray analysis suggested that the non-canonical and canonical Wnt pathways are reciprocally regulated downstream of GATA-2. In the current study, we have identified and characterized a gene encoding the novel protein xTRIL that is upregulated downstream of GATA-2, and that is required for erythropoiesis. In addition to its function during erythropoiesis, xTRIL appears to be necessary and sufficient to activate non-canonical Wnt signaling during convergent extension and other developmental processes. We thus predict that xTRIL activates non-canonical Wnt signaling downstream of GATA-2. We further hypothesize that these events are secondarily required to inhibit canonical Wnt signaling to allow blood progenitors to exit the cell cycle and adopt a hematopoietic fate.

Introduction

In vertebrates, there are two waves of hematopoiesis, termed primitive and definitive hematopoiesis. Definitive hematopoiesis is the process by which multi-potent hematopoietic stem cells (HSCs) give rise to blood cells in all lineages, i.e. erythroid, myeloid and lymphoid cells. Definitive hematopoiesis begins later in embryonic development and continues throughout adult life. In contrast, primitive hematopoiesis is an earlier transient process in which unipotent progenitor cells arise that are capable of producing cells within either the erythroid or myeloid lineage. Definitive hematopoiesis takes place within the embryo proper, while primitive hematopoiesis takes place extra-embryonically in mammalian embryos in the yolk sac blood islands (Galloway and Zon, 2003). In spite of these differences, many of the molecules determined to regulate one wave of hematopoiesis or the other have been shown to be conserved in both processes. Our primary focus of study is in understanding the mechanisms that regulate primitive erythropoiesis. In *Xenopus laevis*, primitive erythropoiesis takes place in an intra-embryonic structure analogous to the mammalian yolk sac blood islands called the ventral blood island (VBI), located on the ventral aspect of the tailbud stage embryo (Galloway and Zon, 2003).

Previous studies have established that primitive erythrocytes require external signals from non-hematopoietic cells in the overlying ectoderm in order to differentiate properly (Kikkawa et al., 2001; Maeno et al., 1994b). A recent series of studies in our lab have subsequently determined that the transcription factor GATA-2 is required in the ectoderm to activate signals that make it

competent to induce blood formation in the mesoderm (Dalgin et al., 2007). To identify GATA-2 targets that might be relevant to primitive erythropoiesis, we used microarray analysis to compare wild type with GATA-2 depleted ectoderm. (Chapter Three). Initial analysis of our microarray data revealed that non-canonical Wnt pathway members are activated downstream of GATA-2 and specifically that Wnt11 is required to promote erythropoiesis. Furthermore, non-canonical Wnts appear to be required to repress canonical Wnt signals to allow for exit from the progenitor state and subsequent erythroid differentiation. These findings have been described in detail in Chapter Three.

In the current study, we describe identification of the *Xenopus* ortholog of the human Toll-like Receptor Interactor with Leucine-rich repeats (xTRIL) as required downstream of GATA-2 in the ectoderm for primitive erythropoiesis. Previously identified functions for this protein are limited to its role in facilitating innate immunity by acting as a co-receptor for Toll-like Receptor 4 (TLR4) (Carpenter et al., 2009). A potential role for TRIL in development has thus not yet been examined. Interestingly, our studies suggest that xTRIL is both necessary and sufficient to activate signaling through the non-canonical Wnt pathway, consistent with xTRIL being activated by GATA-2 upstream of non-canonical Wnts to induce hematopoiesis, and implicating it in the regulation of other non-canonical Wnt functions.

Materials and Methods

Microarray analysis

Microarray analysis was performed as described elsewhere (see Chapter Three).

Embryo culture and manipulation

Ovulation was induced in adult *Xenopus laevis* females by injection of 50 IU of human chorionic gonadotropin (Sigma) into the dorsal lymph sac the night before egg collection in order to induce spawning. Embryos were staged according to Nieuwkoop and Faber (Moon and Christian, 1989; Nieuwkoop and Faber, 1994). Capped synthetic mRNA was synthesized by *in vitro* transcription of linearized template cDNA using a MegaScript kit (Ambion) and injected as indicated in the text and described previously (Moon and Christian, 1989). To assess for defects in convergent extension, Keller explants were performed as described previously (Kuroda et al., 2004). Migration of dorsal cells was also observed by using β-galactosidase (β-gal) as a lineage tracer. Briefly, β-gal mRNA was injected alone or co-injected with xTRIL mRNA into the dorsal midline at the four-cell stage. Embryos were allowed to develop to stage 12 and fixed in MEMFA (0.1 M MOPS, pH 7.4; 2 mM EGTA; 1mM $MgSO_4$; 3.7% formaldehyde) for one hour at room temperature, washed twice in PBS with 1mM MgCl, and washed twice in staining buffer [10 mM $K_3Fe(CN)_6$, 10mM $K_4Fe(CN)_6$, 1mM $MgCl_2$]. Embryos were incubated overnight at room temperature in fresh staining buffer with either 1mg/mL Red-gal (Research Organics), or 1.5mg/mL X-gal (Roche), washed twice in PBS and post-fixed in MEMFA for 30 minutes.

Analysis of RNA

Total RNA was isolated and Northern blots were hybridized with antisense riboprobes as described previously (Christian et al., 1990; von Dassow et al., 1993). Bands were visualized with a phosphorimager and quantified using the NIH ImageJ software. For qRT-PCR analysis, total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions, from which cDNA was generated using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Inc.) with a poly d(T) primer. qRT-PCR was performed using a SYBR Green-based assay (QIAGEN) and a 7900 HT Sequence Detector (ABI). Each sample was analyzed in triplicate and normalized to ODC. Primers used are as follows: *ODC*: F 5′-TGC AGA GCC TGG GAG ATA CT-3′, R 5′-CAT TGG CAG CAT CTT CTT CA-3′; xTRIL: F 5′-ACC AAG TGC TGG CTT TAT CG-3′, R 5′-GGG GAC GAT GAG ACT GAA AA-3'. Embryos were processed for *in situ* hybridization according to the protocol outlined in (Sive et al., 2000).

Analysis of Protein

Cell culture experiments were performed in HeLa cells grown in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For analysis of subcellular localization of xTRIL, HeLa cells were transiently transfected with 2 μg of a FLAG epitope-tagged xTRIL expression plasmid using Lipofectamine 2000 (Invitrogen), and cultured in OptiMEM-I (Invitrogen) for 48 hours. Cells were lysed in modified RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 1 mM PMSF, 1

mM NaF, 1mM Na_3VO_4) with complete protease inhibitor cocktail (Roche), and cleared by centrifugation. Protein concentration was estimated using a BCA assay (Pierce). Proteins were precipitated from conditioned medium using 10% trichloroacetic acid. Proteins present in conditioned medium and cell lysates (50 µg) were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were stained with Ponceau S to confirm equal loading and probed with anti-FLAG antibodies (M2, 1:1000; Sigma). Immunoreactive proteins were detected using the Enhanced Chemiluminescence reagent (Pierce).

To visualize the subcellular localization of xTRIL, xTRIL expression vectors were generated by subcloning full-length *Xenopus* TRIL into $pCS2^+$. HeLa cells were transfected on coverslips, cultured for 24 hours and then fixed in 4% paraformaldehyde for 15 minutes. Cells were permeabilized in 0.2% Triton X-100, then incubated with mouse anti-FLAG antibody (M2, 1:1000; Sigma) in 3% serum overnight at 4^oC. Following three washes with PBS, cells were incubated with Alexa 568-conjugated anti-mouse antibody in 3% FBS for one hour at room temperature, washed three times with PBS, and mounted in Elvanol on slides. Images were acquired with a 63X oil immersion objective lens (NA=1.4) using a Zeiss LSM710 confocal inverted microscope.

For skeletal muscle immunostaining in whole embryos, st. 34 embryos were fixed in Dent's fixative (80% methanol, 20% DMSO) overnight at -20^oC and pigment was bleached by incubation in 1% H_2O_2, 5% formamide, 0.5X SSC for one to two hours on a fluorescent light box. Embryos were washed twice in TBS,

incubated for one hour at room temperature in blocking solution (20% lamb serum in TBS), and then overnight at 4^{o}C in primary antibody (mouse 12/101 monoclonal antibody, 1:100 dilution in blocking solution) followed by hourly washes with TBS for 6 hrs. Secondary antibody incubation (HRP-conjugated goat-anti-mouse at 1:250 dilution) was carried out overnight at 4^{o}C, followed by washes as described above. Signal was detected by incubation in 0.5 mg/ml 3,3'-Diaminobenzidine.

Morpholinos and cDNA constructs

Sequence encoding all but the 5' end of *Xenopus* TRIL was identified by performing BLAST searches of TIGR and Entrez DNA databases. Sequence encoding the N-terminus of *Xenopus TRIL* was obtained by 5' RACE using oligonucleotide primers complementary to sequence near the 5' end of the known sequence. RNA was isolated from *Xenopus* embryos at stage 34, reverse transcribed and used as a template for PCR-mediated amplification of a *xTRIL* cDNA (GenBank number *to be submitted*) that encodes the entire open reading frame, as determined by alignment with *xTRIL* from other species (not shown). A morpholino antisense oligonucleotide (MO) complimentary to both alleles of *xTRIL* (xTRIL MO: 5'-CCA AAA TCT GGG CAT CAC CTT TC-3') was purchased from Gene Tools, LLC (Philomath, OR). Sequence encoding a FLAG epitope tag was appended to the 5' end of the *xTRIL* open reading frame by PCR-mediated amplification. All cDNAs were subcloned into pCS2+ for RNA transcription and transient transfection of mammalian cells.

Results

Expression of the novel protein, xTRIL, is induced downstream of GATA-2 in the ectoderm

As described previously (Chapter Three), we used microarray analysis to identify targets of GATA-2 in the ectoderm, which could mediate signals required for erythropoiesis in the mesoderm. In this screen, we identified a novel gene that encodes a putative trans-membrane protein and was strongly upregulated in GATA-2 RNA injected ectoderm (+9.6-fold) and downregulated in both GATA-2 morphant and FOG RNA injected ectoderm (-6.1 and -3.2 fold, respectively). Database searches subsequently identified this target as the *Xenopus* ortholog of a gene recently identified in studies of the human innate immune system, *Toll-like receptor interactor with leucine-rich repeats* (*TRIL*), hereafter referred to as *xTRIL*. In the immune system, TRIL has been shown to interact with Toll-like receptor4 (TLR4) and to be necessary and sufficient to enhance TLR4-mediated cytokine production (Carpenter et al., 2009). xTRIL is well-conserved at the N- and C- termini, bearing homology to chicken, mouse and human orthologs, though its expression or function outside of innate immunity has not yet been examined.

Expression of xTRIL is strongly induced by GATA-2 in the ectoderm

In order to validate the changes in xTRIL expression predicted by the microarray analysis, we performed qRT-PCR on ectodermal explants from

embryos that had been injected with GATA-2 MO or FOG RNA at the two-cell stage. Explants were taken at the gastrula stage (st. 10), cultured to stage 12 and harvested for RNA. As predicted by our microarray analysis, expression of *xTRIL* in the ectoderm was strongly repressed by both GATA-2 MO and FOG RNA injection (Fig. 4-1A). To determine whether regulation of xTRIL by GATA-2 is due to a cell-autonomous mechanism, FOG RNA was injected into only one of two cells at the two-cell stage. Embryos were allowed to develop to stage 14 and *xTRIL* expression was assessed by *in situ* hybridization. As shown in Fig. 4-1B-D, TRIL transcripts were detected in the ventrolateral ectoderm at stage 14 (purple arrows). Compared with uninjected controls, we observed unilateral

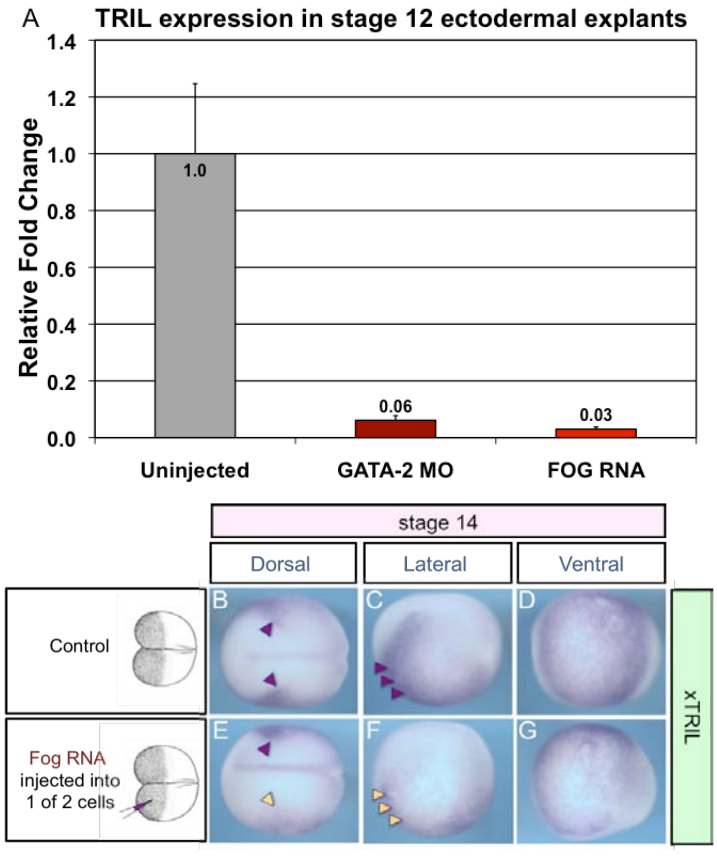


Figure 4-1. Expression of xTRIL is reduced in GATA-2 deficient embryos. (A) Both blastomeres of embryos at the two-cell stage were injected with either GATA-2 MO or FOG RNA. Ectoderm was explanted at stage 10, and cultured to stage 12 when RNA was harvested and expression of *xTRIL* was assessed by qRT-PCR analysis to determine changes in expression of *xTRIL*. (B-G) Control embryos (B-D) or embryos in which FOG RNA was injected into one of two cells at the two-cell stage (E-G), were allowed to develop to stage 14 and assessed for changes in *xTRIL* expression by *in situ* hybridization. Anterior is to the right in all cases.

reduction of *xTRIL* expression in FOG RNA injected embryos indicating that xTRIL is downregulated by disruption of GATA-2 in a cell-autonomous fashion.

xTRIL is expressed at the gastrula stage in ventrolateral ectoderm and dorsal mesoderm

To begin to ask if xTRIL plays a role in primitive erythropoiesis, we wished to determine whether it is expressed in the right time and place to do so. Using whole mount *in situ* hybridization, we characterized the expression pattern of xTRIL throughout early *Xenopus* development beginning at the four-cell stage and continuing through the late tailbud stage (Fig. 4-2A-N and data not shown). Expression of xTRIL was first detectable by Northern blot analysis (data not shown) and whole-mount *in situ* hybridization at the onset of gastrulation (stage 10). At this stage, xTRIL was strongly expressed in the ventrolateral ectoderm (VE) and dorsal mesoderm (DM) (Fig. 4-2A, B) and expression persisted in these areas through stage 12 (Fig. 4-2C-F). xTRIL is thus expressed at precisely the right time (gastrulation) and in the right location (ventral ectoderm) to mediate ectodermal signals required for blood. Further analysis of xTRIL expression through the neurula and tailbud stages indicated that it continues to be expressed in the dorsal mesoderm (notochord, not shown) and ventral ectoderm through stage 19 (Fig. 4-2H, I, K) as well as in pre-migratory neural crest cells (Fig. 4-2J, L), somites, pharyngeal arches (Fig. 4-2M, N) and the tailbud (Fig. 4-2N). The function of xTRIL in these tissues is an interesting avenue that will be addressed in future studies.

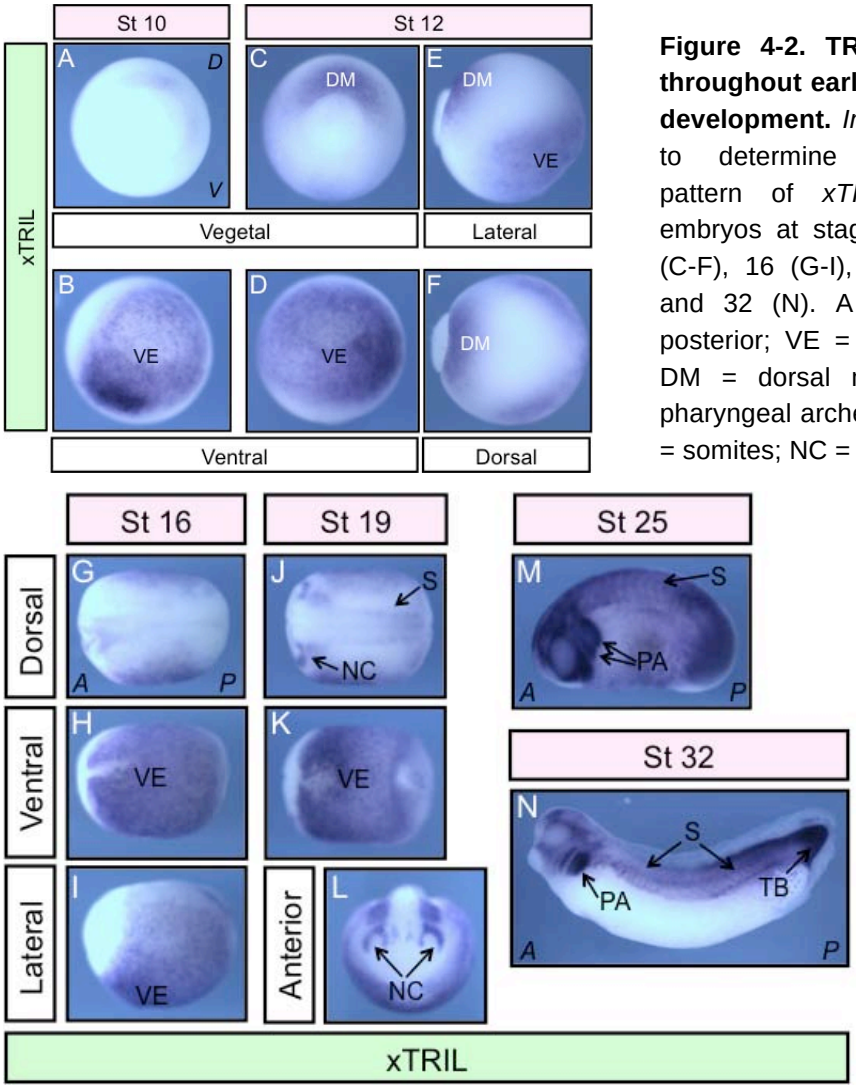


Figure 4-2. TRIL is expressed throughout early *Xenopus laevis* development. *In situ* hybridization to determine the expression pattern of *xTRIL* in *Xenopus* embryos at stages 10 (A, B), 12 (C-F), 16 (G-I), 19 (J-L), 25 (M) and 32 (N). A = anterior; P = posterior; VE = ventral ectoderm; DM = dorsal mesoderm; PA = pharyngeal arches; TB = tailbud; S = somites; NC = neural crest.

xTRIL is localized to the membrane and to intracellular vesicles in HeLa cells

As xTRIL is highly expressed in ventral ectoderm during gastrulation, we wished to determine if it was capable of mediating a signal between the ectoderm and mesoderm either by being secreted or through anchorage to the membrane and extension into the extracellular space. Structural predictions based on the amino acid sequence indicate that xTRIL has a putative transmembrane domain (Fig. 4-3A). In addition, a previous report has cited preliminary data indicating

that certain xTRIL isoforms may be secreted (Carpenter et al., 2009). We therefore generated an epitope-tagged version of xTRIL bearing a FLAG tag at the C-terminus, transfected it into HeLa cells and asked whether we could detect xTRIL protein within intracellular and/or extracellular fractions by Western blotting (Fig. 4-3B). Although xTRIL protein was detectable by Western blotting in cell lysates, it was absent in the media (Fig. 4-3B), suggesting that it is not secreted, at least in this system. As an alternate approach to the same question, we also used immunocytochemistry to visualize the localization of xTRIL in transfected

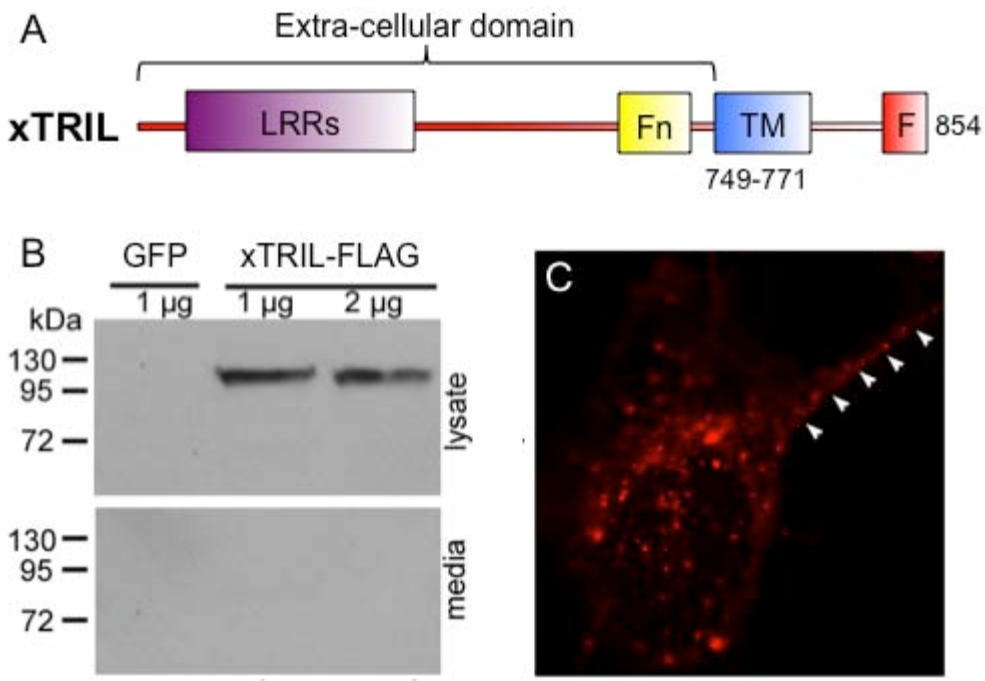


Figure 4-3. xTRIL is localized to the cell membrane and to intracellular punctae. (A) Schematic of xTRIL depicting the extra-cellular Leucine-rich repeat domain (LRRs), Type III Fibronectin domain (Fn), transmembrane domain (TM) and FLAG epitope tag (F) at C-terminus. (B) HeLa cells were transiently transfected with either one or two micrograms of xTRIL-FLAG or one microgram of GFP DNA as a control. Cell lysates and media were harvested separately after 24 hours and protein was analyzed by Western blotting for the FLAG epitope to determine expression of xTRIL. (C) HeLa cells transfected as described in (B) were immunostained with an anti-FLAG antibody and visualized by confocal microscopy to detect subcellular localization of xTRIL protein. White arrowheads indicate membrane localized xTRIL.

HeLa cells (Fig. 4-3C, D). Consistent with these findings, confocal imaging of fluorescently labeled xTRIL protein demonstrated that xTRIL is present in discrete subcellular punctae as well as on the cell surface (Fig. 4-3D). Together, these findings suggest that xTRIL is present on the cell membrane and in intracellular vesicles but not secreted. However, we cannot rule out the possibility that an extracellularly cleaved form of xTRIL is generated, since our epitope tag is located at the C-terminus of the protein. Additional studies using xTRIL-specific antibodies or an N-terminally tagged version of xTRIL will allow us to more definitively resolve this question.

Ectodermal xTRIL is required for primitive erythropoiesis

Our above data indicate that xTRIL is positively regulated downstream of GATA-2 and is expressed in precisely the right cells, during the appropriate developmental window to be involved in primitive erythropoiesis. To begin to test whether or not xTRIL is required for RBC development, we designed a translation-blocking antisense morpholino oligonucleotide (MO) against xTRIL (see Methods for additional details). xTRIL MO was injected at the two- to four-cell stage at doses ranging from 20-80ng, and expression of *globin* was assessed at the tailbud stage by Northern blotting (Fig. 4-4). Embryos injected with the control MO showed expression of *globin* at levels comparable to uninjected embryos (Fig. 4-4). In contrast, xTRIL MO injected embryos showed dramatically reduced expression of *globin* in a dose-dependent fashion (Fig. 4-4). Additional rescue experiments will be necessary to confirm specificity of the MO.

However, these preliminary data are consistent with the hypothesis that xTRIL activation downstream of GATA-2 in the ectoderm is required for primitive erythropoiesis.

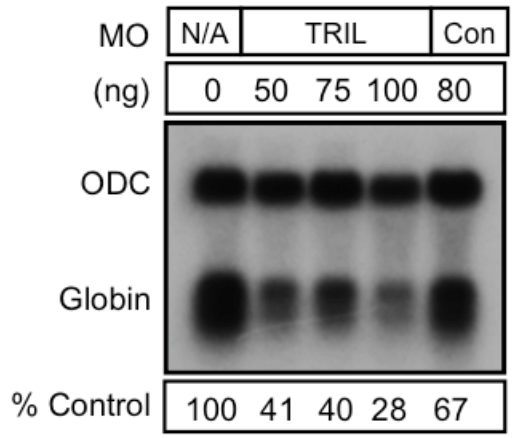


Figure 4-4. xTRIL morphants show a dose-dependent loss of *globin* expression at the tailbud stage. Control MO (Con) or xTRIL MO, in doses ranging from 50 to 100ng, were injected into the animal pole at the two- to four-cell stage and expression of *globin* was analyzed by Northern blotting at stage 34. Levels of *globin* transcripts are normalized relative to expression of *ODC* and reported as a percentage of control below each lane.

Ectopic expression of xTRIL in dorsal cells blocks convergent extension without affecting cell fate

As a preliminary assay to determine which signaling pathways might be activated downstream of xTRIL during *Xenopus* embryogenesis, we injected RNA encoding xTRIL near the dorsal or ventral midline of embryos at the four-cell stage and looked for patterning defects that phenocopy those caused by aberrant activation of known developmental signaling pathways. We found that injection of xTRIL into ventral cells, where it is normally expressed (Fig. 4-2), had no discernible effect on morphology (Fig. 4-5B compared with A). Strikingly, ectopic expression of xTRIL near the dorsal midline produced embryos that showed a drastically shortened anterior/posterior (A/P) axis and failure of the neural folds to fuse, resulting in *spina bifida* (Fig. 4-5C). On a gross level, these

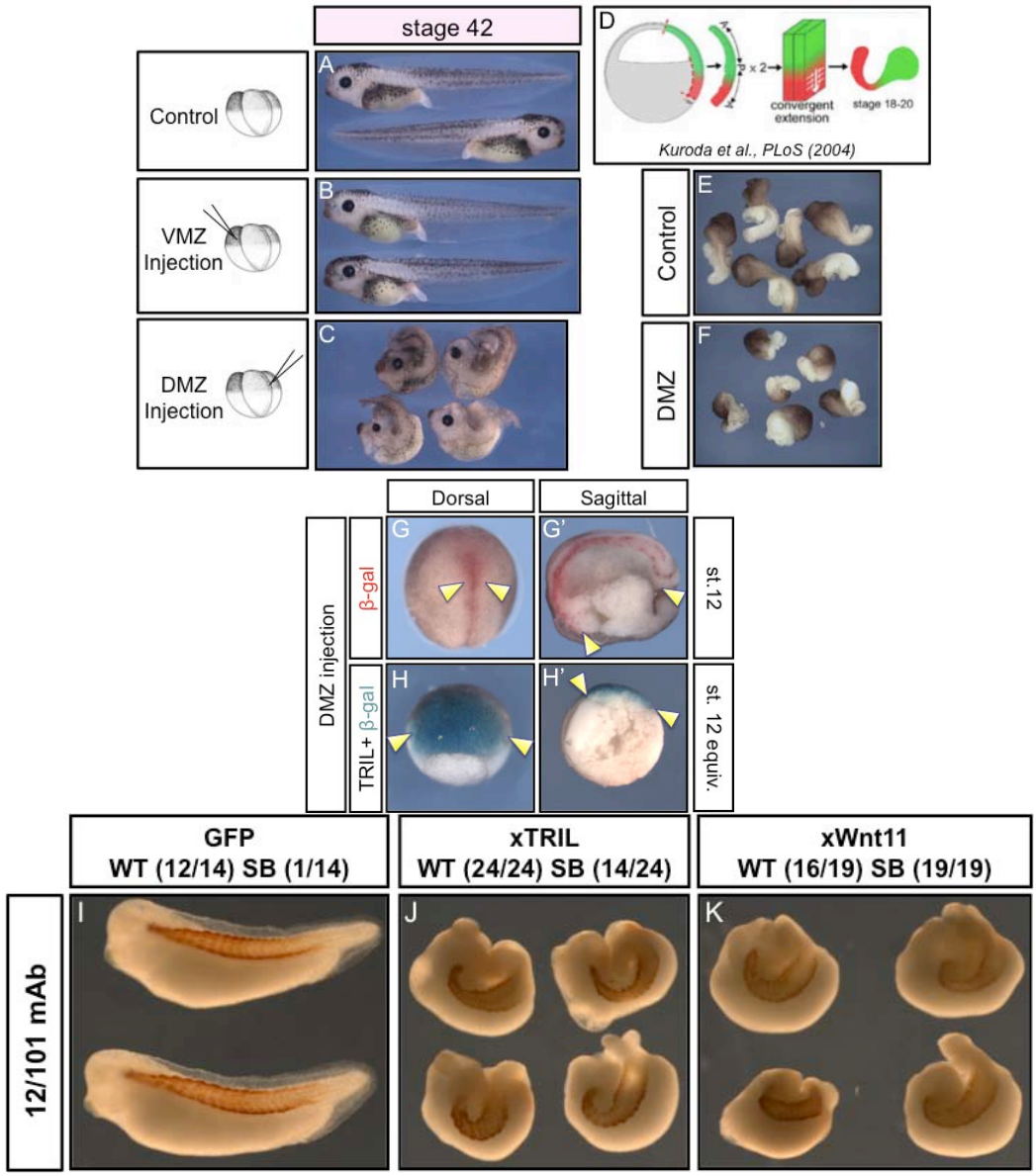


Figure 4-5. Overexpression of xTRIL in dorsal cells blocks convergent extension without affecting cell fate, phenocopying hyperactivation of the non-canonical Wnt pathway. Control embryos (A), or embryos injected with xTRIL mRNA near the VMZ (B) or DMZ (C) at the four-cell stage were allowed to develop to the tadpole stage (stage 42), and evaluated for morphological defects. Keller explants (schematized in D) were performed to assess for convergent extension defects using uninjected embryos (E) or embryos that had been injected with xTRIL mRNA near the dorsal midline (F). Explants were taken at the gastrula stage and allowed to develop to stage 18-20 for evaluation. (G-H) β-galactosidase (β-gal) mRNA was injected as a lineage tracer either alone (G, G') or with xTRIL mRNA (H, H') into the dorsal midline at the four-cell stage. Localization of β-gal injected cells was visualized at stage 12 (blue or red stain) in whole embryos (H, G) or mid-saggital sections (H', G'). Yellow arrowheads indicate migration of dorsally derived cells to the midline (G, H) or involution and migration of dorsal cells during gastrulation (G', H'). (I-K) Embryos were injected with GFP control (I), xTRIL (J), or xWnt11 (K) mRNA near the DMZ at the four-cell stage, allowed to develop to the tailbud stage (st. 32) and immunostained with 12/101, a marker of skeletal muscle. GFP=green fluorescent protein; WT=wild type; SB=*spina bifida*.

defects resemble those observed in embryos in which convergent extension (CE) movements of the dorsal mesoderm and ectoderm are disrupted (Wallingford et al., 2002b). CE is a highly regulated vertebrate process in which polarized intercalation and migration of cells toward the embryo midline during and after gastrulation results in axis elongation and fusion of the neural folds on the dorsal side.

To more stringently test for defects in CE, we employed several established assays. First, we used the Keller explant assay in which dorsal marginal zone (DMZ) explants are dissected at the gastrula stage (st. 10), cultured to the neurula stage (st. 19) and assessed for their ability to subsequently undergo convergence, which drives elongation (schematized in Fig. 4-5D). We found that DMZ explants from wild type embryos narrowed and elongated normally (Fig. 4-5E), whereas those that overexpressed xTRIL RNA remained wide and failed to elongate (Fig. 4-5F), indicative of CE defects. As a second assay for defects in CE, we injected β-galactosidase (β-gal) mRNA either alone or together with xTRIL RNA into dorsal cells of embryos at the four-cell stage. These embryos were allowed to develop through gastrulation to stage 12, at which time embryos were stained for β-gal activity. In wild type embryos, dorsal cells converged to the midline, forming a thin stripe of β-gal positive cells (Fig. 4-5G, yellow arrowheads). In contrast, embryos co-injected with β-gal and xTRIL showed β-gal positive cells in a broad band on the dorsal side, indicating that they had failed to migrate to the midline (Fig. 4-5 H, yellow arrowheads). In addition, these embryos underwent abnormal gastrulation movements as

demonstrated by failure of the dorsal cells to properly invaginate into the embryo, evident in mid-saggital sections of the same embryos shown in Fig. 4-5 G and H. (Fig. 4-5H' compared with 4-5G', yellow arrowheads). Together, these results demonstrate that overexpression of xTRIL causes defects in CE and suggest that ectopic expression of xTRIL perturbs cell movement as opposed to cell fate determination.

To verify that dorsal cell populations are present in embryos made to overexpress xTRIL, injected embryos were stained at the tailbud stage with 12/101, a skeletal muscle specific antibody (stage 34; Fig. 4-5I, J). If xTRIL overexpression disrupts cell fate specification, we would expect dorsally-derived structures such as muscle and notochord to be absent or reduced. While the pattern of 12/101 staining was somewhat distorted reflecting the defects in cell movement, the overall intensity of staining and chevron shaped patterning of the somites was intact (Fig. 4-5J, compared to uninjected controls in Fig. 4-5I). Moreover, embryos in which Wnt/PCP signaling was upregulated by injection of RNA encoding the non-canonical Wnt ligand, Wnt11, showed defects that were indistinguishable from those induced by overexpression of xTRIL (Fig. 4-5K). Together, these data demonstrate that xTRIL specifically affects CE movements independent of cell fate and suggest that xTRIL can activate the non-canonical Wnt pathway when overexpressed.

xTRIL morphants show *cardia bifida*, similar to embryos in which non-canonical Wnt signaling is inhibited

The above results raise the possibility that xTRIL can activate non-canonical Wnt signaling *in vivo* and thus we examined xTRIL morphants for defects observed in embryos deficient in non-canonical Wnt signaling (Abu-Elmagd et al., 2006; De Calisto et al., 2005; Pandur et al., 2002). Using *in situ* hybridization, we examined markers of blood (*globin*), heart [*cardiac troponin* (*TnC*)] and neural crest [*Distalless 2* (*Dll2*)] cells (Fig. 4-6A, B). Confirming our previous Northern analysis (Fig. 4-4), and consistent with a defect in non-canonical Wnt signaling (discussed in Chapter Three) *globin* expression was reduced in xTRIL morphants (Fig. 4-6A, B orange arrow heads). Interestingly, xTRIL morphants also exhibited cardia bifida (Fig. 4-6A, B yellow arrow head), a failure of the left and right heart fields to fuse, which is similar to defects seen in embryos deficient in non-canonical Wnt signaling (Garriock et al., 2005; Matsui et al., 2005). Although non-canonical Wnts have been implicated in neural crest development (Abu-Elmagd et al., 2006; De Calisto et al., 2005), and xTRIL is

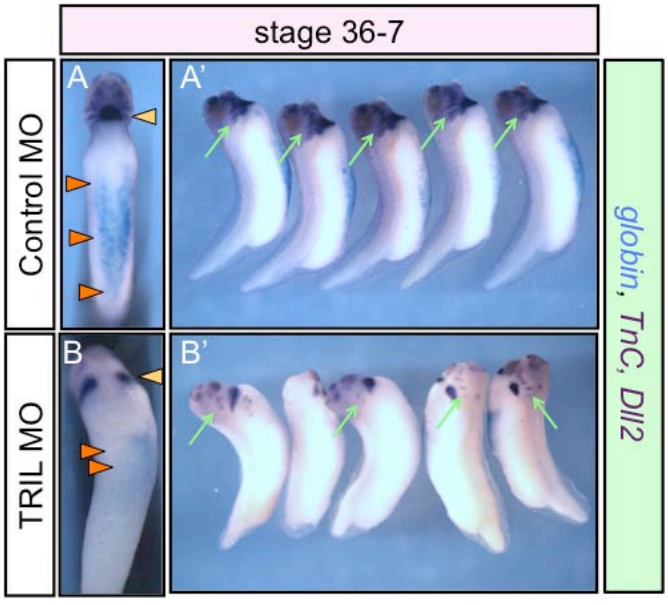


Figure 4-6. xTRIL morphants show decreased *globin* and *Dll2* expression, and cardia bifida. Control embryos (A, A') or embryos injected with xTRIL MO at the two-cell stage (B, B') were allowed to develop to the tailbud stage (st. 36-7) and assessed for expression of *globin* (orange arrowheads), *Distalless 2 (Dll2*; green arrows), and *cardiac troponin* (*TnC*; yellow arrowheads) by *in situ* hybridization.

expressed in pre-migratory neural crest cells (Fig. 4-2L), expression of *Dll2* is variably reduced in xTRIL morphants (Fig. 4-7A, B green arrows). Therefore, though this non-canonical Wnt regulated process may also be affected by xTRIL, further analysis is required to conclusively answer this question. Collectively, our analysis of xTRIL overexpressing and morphant embryos is consistent with the hypothesis that xTRIL is required to activate the non-canonical Wnt signaling pathway downstream of GATA-2 *in vivo*.

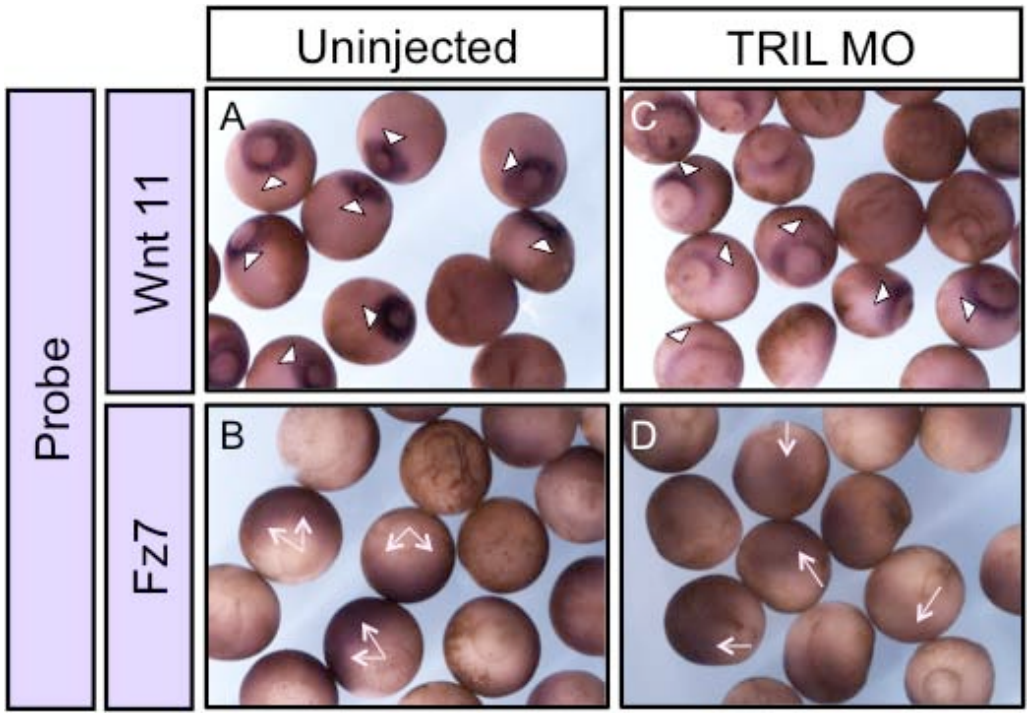
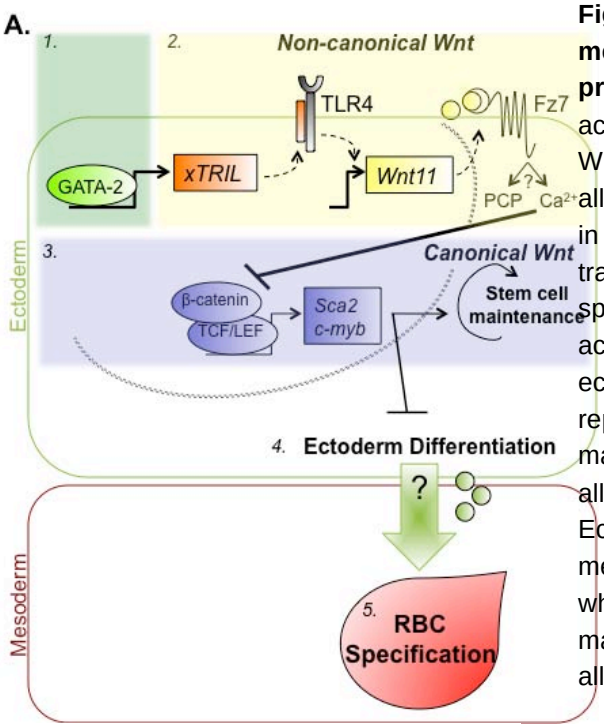


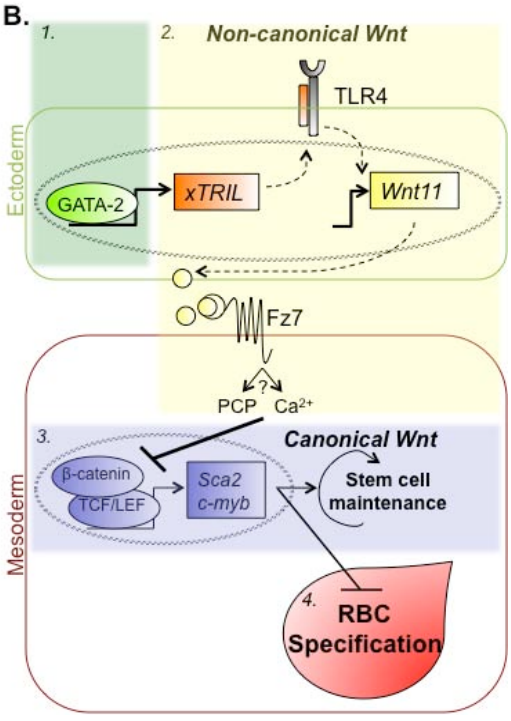
Figure 4-7. xTRIL regulates expression of non-canonical Wnt pathway members during gastrulation. Uninjected controls (A, B) or embryos injected with TRIL MO at the two-cell stage (C, D) were allowed to develop to stage 12 and *in situ* hybridization was performed to determine expression of *Wnt11* (A, C, white arrowheads) or *Fz7* (B, D white arrows).

xTRIL regulates expression of non-canonical Wnt pathway members

Our microarray analysis identified both xTRIL and non-canonical Wnts as activated downstream of GATA-2 in the ectoderm. Moreover, data from this study

and our previous study indicate that both xTRIL and non-canonical Wnt signaling are required for primitive erythropoiesis. Finally, the above data clearly demonstrate that xTRIL likely functions to promote signaling through the non-canonical Wnt pathway. However, the hierarchy by which these signals act to regulate primitive erythropoiesis downstream of GATA-2 has not yet been established. As both Wnt11 and Frizzled7 (Fz7) were identified as positively regulated by GATA-2 in our microarray analysis, and because we have previously established a role for Wnt11 in regulating primitive erythroid development, we began by assessing whether expression of these non-canonical Wnt pathway members is altered upon depletion of xTRIL. Specifically, embryos were injected at the two-cell stage with xTRIL MO and expression of Wnt11 and Fz7 was analyzed by *in situ* hybridization at stage 12. We find that depletion of xTRIL resulted in reduced expression of both Wnt11 and Fz7 (Fig. 4-7, white arrowheads and arrows), suggesting that xTRIL functions as an upstream activator of the non-canonical Wnt signaling pathway. Collectively, these data are consistent with a model in which ectodermal GATA-2 activates expression of xTRIL, which in turn activates expression of the non-canonical Wnt pathway members Wnt11 and Fz7. Based on our previous data, we further hypothesize that non-canonical Wnt signals are then required to repress canonical Wnt targets to allow erythroid precursors to exit from the progenitor state and differentiate into mature red blood cells (see Fig. 4-8 for a model).





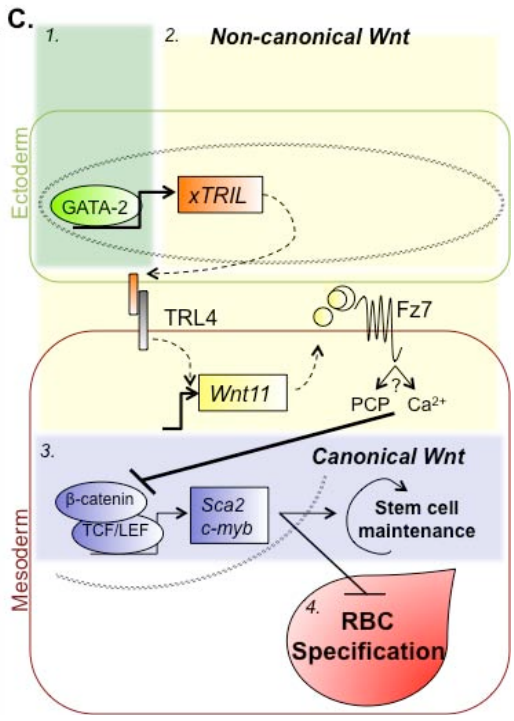


Figure 4-8. Model for potential mechanism of xTRIL regulation of primitive erythropoiesis. (A) xTRIL activates expression of non-canonical Wnts, which repress canonical Wnts, allowing for ectoderm differentiation. This in turn allows for development and transmission of signals that support RBC specification in the mesoderm. (B) xTRIL activates non-canonical Wnts in the ectoderm, which signal to the mesoderm to repress hematopoietic progenitor maintenance by canonical Wnts and allows for RBC specification. (C) Ectodermal xTRIL signals directly to the mesoderm to activate non-canonical Wnts, which represses hematopoietic progenitor maintenance by canonical Wnt signals to allow for RBC specification.

Discussion

In the current study we have used microarray analysis to identify a role for the novel gene, xTRIL, in regulating primitive erythropoiesis downstream of GATA-2 in the ectoderm. Moreover, we have shown that xTRIL is both necessary and sufficient to signal through the non-canonical Wnt pathway. Our previous studies demonstrate that GATA-2 is required to reciprocally regulate non-canonical and canonical Wnt signaling in the ectoderm to support normal erythropoiesis (Chapter Three). Together with these findings our current study is consistent with the hypothesis that in the ectoderm, GATA-2 mediated activation of xTRIL results in secondary activation of the non-canonical Wnt pathway, possibly via induction of expression of Wnt11, the non-canonical ligand identified in our prior study as essential for RBC development (Chapter Three). We further hypothesize that non-canonical Wnt signaling is required to dampen canonical Wnt signals to permit RBC differentiation in the mesoderm (see Fig. 4-8 for a proposed model).

As these data constitute preliminary studies, several outstanding questions still remain to be addressed. For example, we have not yet determined whether xTRIL is a direct target of GATA-2 or is secondarily activated by another intermediate. Initial sequence analysis of the promoter region of xTRIL indicates that it is enriched for WGATAR motifs (not shown), suggesting that it may be directly activated by GATA-2. Additional molecular studies, such as reporter and *in vitro* binding assays, will be required to definitively address this question.

In other initial studies, we would like to determine if xTRIL is required in the ectoderm, the mesoderm or in both tissues during hematopoiesis. To address this question, we will use MOs to ask if selective depletion of xTRIL in either the ectoderm or the mesoderm affects RBC specification and/or differentiation in recombinants. Based on its pattern of expression in whole embryos at the gastrula stage, we predict xTRIL is specifically required in the ectoderm for primitive erythropoiesis. However, as we have not yet performed *in situ* analysis on sectioned embryos, we cannot rule out the possibility that xTRIL is also expressed at low levels in deep mesodermal cells and that it may function there as well.

While we our data suggest that xTRIL is capable of activating non-canonical Wnt signaling, we have not determined whether it does so up or downstream of ligand-receptor interactions. Although our initial epistasis study indicates that xTRIL may be acting upstream of non-canonical pathway members, it does not rule out the possibility that both xTRIL and non-canonical Wnts are acting in parallel pathways to support a process (such as general ectoderm differentiation) required to maintain normal expression of both xTRIL and Wnts. Further studies to validate this relationship include using qPCR or Northern blotting to confirm the reduction in canonical Wnt pathway members observed upon depletion of xTRIL by *in situ*, as well as performing the reciprocal analysis to verify that loss of non-canonical Wnts does not disrupt expression of xTRIL.

In more long-term studies, we are interested in exploring the other potential signaling mechanisms associated with xTRIL during development. Human TRIL (hTRIL) was previously identified in a microarray screen for novel Lipopolysaccharide (LPS)-inducible genes in embryonic stem (ES) cells. This study demonstrated that hTRIL is both necessary and sufficient for LPS-induced cytokine expression mediated by the Toll-like Receptor 4 (TLR4) signaling pathway (Carpenter et al., 2009). Determining whether there is similarity between the hTRIL-activated pathway in immunity and xTRIL's function during red blood cell development is a point of interest. The TLR4 pathway is known to act via stabilization of an NFκB intermediate (Doyle and O'Neill, 2006), thus one way we have begun to address this possibility is by asking if inhibiting NFκB during gastrulation affects RBC differentiation. These studies are intended to give us a preliminary indication of whether xTRIL signals via a similar mechanism in both contexts, which would be of interest for future studies.

Given that xTRIL is expressed in many different tissues during development and gives multiple loss of function phenotypes, we predict that it has additional functions distinct from its role in erythropoiesis that remain to be discovered. Indeed overexpression and loss-of-function of xTRIL respectively replicates gain- and loss-of-function phenotypes of non-canonical Wnt signaling, indicating that xTRIL is likely involved in other processes that rely on the Wnt pathway, such as heart and axis development. Exploring these potential functions of TRIL in both *Xenopus* and mouse will be the subject of future studies.

CHAPTER 5

Conclusions & Future Directions

The work presented herein aims to understand how formation of primitive red blood cells is regulated during development by signals both within hematopoietic precursors, as well as by supportive signals from the local environment. In the case of *Xenopus laevis* these requisite external signals are provided by the overlying ectoderm. Studies described in Chapter Two have identified a requirement for the transcriptional co-factor, *Friend of GATA* (*FOG*) during *Xenopus* primitive erythropoiesis and thereby resolved conflicting data in the literature by demonstrating that similar to its role in other vertebrates, FOG is required for blood development in frogs. Moreover, we showed that overexpression of FOG has a dominant negative effect on erythropoiesis, resulting in loss of blood that is unrelated to its normal function. These studies have succeeded in bringing the role of FOG in *Xenopus* in line with what is known about its function during erythropoiesis in both mice and humans, thus reaffirming the use of frogs as a relevant model system in which to study hematopoietic regulation in higher vertebrates. In addition, using structure-function analysis, we have identified a novel requirement for the Nucleosome Remodeling and Deacetylase (NuRD) complex interaction domain of FOG during blood development.

Although the role of FOG is well studied within hematopoietic cells, a potential role in non-hematopoietic cells has not yet been tested. Previous data from our lab demonstrating that GATA-2 is required in ectodermal cells for primitive erythropoiesis, together with data presented in Chapter Three which show that overexpression of FOG has a dominant-negative effect on GATA-2

function in these cells, lead us to hypothesize that FOG may also function in ectoderm as a required cofactor for GATA-2. The studies described in Chapter Two will therefore be complemented by future analysis in which we will examine a non-cell autonomous requirement for FOG. Should we find that ectodermal FOG is in fact required for erythropoiesis, we will also take advantage of the mutant constructs already developed to determine which domains of FOG and potential interaction partners are relevant to this putative role.

While the requirement for non-cell autonomous signals from adjacent tissues is a well-conserved phenomenon, previous attempts to uncover the identity of this signal in multiple systems have been unsuccessful. Based on prior work in the lab, we knew that ectodermal GATA-2 was required for primitive erythropoiesis. However, genes downstream of GATA-2 that could practically mediate a signal between ectoderm and mesoderm were unknown. We thus turned to microarray analysis as an unbiased method to identify GATA-2 targets that might be relevant for hematopoiesis (described in Chapters Three and Four).

As the *Xenopus laevis* genome is not fully sequenced and annotations for the Affymetrix array chip are incomplete, analysis of microarray data in *Xenopus laevis* produced some unique challenges. In addition, because gene regulation in early embryonic development is exceedingly complex due to the condensed timing of events and the sheer number of genes being coordinately activated and repressed, we found it necessary to precisely control the parameters of our input samples to minimize background (see Appendix for more detail on performing microarray studies in *Xenopus laevis*). Moreover, *Xenopus laevis* is not an

isogenic model system; one might therefore reason that the high degree of background common to microarray experiments might also pose a challenge. However, we believe that this has instead worked to our advantage by imposing a higher level of stringency on our analysis. In other words, only robust changes in gene expression that are not easily perturbed by variation in genetic background appear significantly altered by our injection conditions. This has been borne out by subsequent validation of many of our targets using a variety of methods.

As presented in Chapter Three, we identified the non-canonical and canonical Wnt pathways as being reciprocally regulated downstream of GATA-2, a relationship which has been described for heart and lung development, but which is a novel discovery with regard to hematopoiesis. In addition, we identified the *Xenopus* ortholog of Toll-like Receptor Interactor with leucine-rich repeats (xTRIL) as being highly activated downstream of GATA-2 (Chapter Four). Identification of this protein was particularly exciting given that in addition to its role in erythropoiesis, xTRIL was also found to activate non-canonical Wnt signaling. This observation nicely tied its function to the Wnt-related trends predicted by our microarray, while simultaneously opening up many new avenues of research, by implicating a role for xTRIL in additional developmental processes regulated by the Wnt pathway.

The more immediate goals for this project include key epistasis studies that would definitively establish the order of signaling events downstream of GATA-2, as well as studies to more precisely define which events are specific to

the ectoderm and which are specific to the mesoderm (described in detail in the Discussions for Chapters Three and Four). Additional long-term directions that are of interest relate to the potential developmental functions and signaling mechanisms of xTRIL, which are relatively unknown.

Based on xTRIL loss-of-function phenotypes as well as on the expression patterns identified by *in situ* analysis in *Xenopus* (presented in Chapter Four), and in preliminary mouse studies (not shown), we expect that xTRIL has developmental functions outside of hematopoiesis. It would therefore be of interest to further explore these additional roles by knocking out TRIL function in the mouse to validate observed hematopoietic defects and to determine if defects in organs that depend upon Wnt signaling are also observed.

Although little is known about TRIL function during development aside from what has been described herein, it has been shown to be both necessary and sufficient to activate signaling through Toll-like receptor 4 (TLR4) in a cell culture model of innate immunity (Carpenter et al., 2009). The TLR4 pathway is comparatively well characterized and is generally thought to signal via NFκB (Doyle and O'Neill, 2006). Downregulation of NFκB has been shown by others to be required for differentiation of erythrocytes during both embryonic and adult hematopoiesis, however its expression or function during erythroid commitment has not been examined. Interestingly, expression of XrelA, the NFκB subunit in *Xenopus*, peaks at gastrulation in whole embryos (Richardson et al., 1994). This is the same developmental timeframe that we identified during which ectodermal signals are necessary for erythroid commitment. Determining whether or not

TRIL modulates NFκB signaling to promote erythroid commitment would thus be an interesting future direction of study.

The possible involvement of Toll-like receptor signaling in vertebrate development reminds one with an active imagination of the molecule for which these receptors were named, Toll. The Toll/Dorsal pathway was first identified in *Drosophila* development as essential for establishing the dorsal/ventral axis in the early development (Rushlow et al., 1989) as well as a regulator of innate immunity in flies (Gordon et al., 2005). Additional studies have identified the WntD ligand as a transcriptional target of this pathway (Ganguly et al., 2005; Gordon et al., 2005). Based on homology to other Wnts, WntD has not yet been assigned to either the non-canonical or canonical pathway. An attractive theory is that TRIL could activate the Toll/Dorsal pathway by serving as a co-receptor (similar to its function in TLR4 signaling) for a vertebrate Toll ortholog. This could in turn activate a non-canonical Wnt ligand, such as Wnt11, required for hematopoiesis and other developmental events.

Finally, in addition to the genes associated with the Wnt pathway and to xTRIL, a very large number of genes required for cilia formation were also identified by our microarray analysis as positively regulated downstream of GATA-2. Currently, we have two hypotheses that might account for this observation. The first is based on studies demonstrating that non-canonical Wnt signals are required for establishing planar cell polarity during epithelial development (Wallingford et al., 2002a). Epithelial polarity is in turn required for proper cilia formation and orientation. Disruptions in non-canonical Wnt signaling

could thus indirectly cause defects in the regulation of ciliary genes. A second hypothesis, which is also supported by our microarray analysis, is that in addition to its function in erythropoiesis, GATA-2 also controls expression of a master transcriptional regulator of ciliary genes, FoxJ1 (Stubbs et al., 2008; Yu et al., 2008). Loss of FoxJ1 expression would be consistent with the downregulation of numerous cilia-related genes that we observe in our microarray analysis upon depletion of GATA-2. Should this hypothesis be correct, it would suggest that ectodermal GATA-2 does much more than simply regulate hematopoiesis, and that it may in fact be involved in overall ectoderm maturation and development. This idea would also be consistent with the fact that a straight-forward search for the “single downstream target” of GATA-2 required for erythropoiesis has instead yielded a wealth of interesting intermediate signals and pathways that have only resulted in more complex and exciting questions.

APPENDIX

Manipulation of gene function in *Xenopus laevis*

Mizuho S. Mimoto and Jan L. Christian

Department of Cell and Developmental Biology

Oregon Health and Science University, School of Medicine

3181 SW Sam Jackson Park Road

Portland OR 97239-3098

To be published in *Methods in Molecular Biology, Vertebrate embryogenesis: Methods and Protocols* (Humana Press, Springer Publishing Group; Ed. Francisco J. Pellegri, Ph.D., series editor John Walker, Ph.D.)

Abstract
Xenopus laevis embryos are particularly well suited to address questions requiring either knockdown or overexpression of genes in a tissue-specific fashion during vertebrate embryonic development. These manipulations are achieved by targeted injection of either antisense morpholino oligonucleotides, or synthetic mRNAs, respectively, into the early embryo. Herein we offer detailed protocols describing how to design and perform these experiments successfully, as well as a brief discussion of considerations for performing a microarray analysis in this organism.

Key Words: *Xenopus laevis*; embryogenesis; microinjection; morpholinos; gene knockdown; microarray

1. Introduction

Among the many advantages of working with *Xenopus laevis* embryos as compared with other vertebrate embryos are that they are very large (approximately 1mm in diameter at the one-cell stage), and they develop rapidly and externally. Their size and accessibility allow for microdissection and manipulation of specific tissues at even the earliest stages of development. In addition, their characteristic pigmentation and cleavage patterns together with extensive lineage tracing studies (Dale and Slack, 1987; Moody, 1987a; Moody, 1987b), facilitate targeted injection of constructs in order to manipulate early gene expression in a tissue specific fashion (*see* Fig. A-1). Individual blastomeres may be targeted reasonably well up to the 32-cell stage. For later stage targeting, it is preferable to use an alternate strategy, such as transgenesis employing the appropriate DNA promoter, to achieve overexpression in a regulated fashion. This technique is described in an accompanying chapter, as is the use of chemicals in order to induce competency in specific tissue types (e.g. mesoderm induction by Animal Cap exposure to Activin (Green et al., 1994)), to perturb specific signaling pathways (e.g. inhibition of FGF signaling by incubation in SU5402 (Galli et al., 2003)) or to alter cell fate (e.g. UV or LiCl treatment to ventralize or dorsalize embryos, respectively (Kao et al., 1986; Scharf and Gerhart, 1983)) all of which are common approaches used to study changes gene expression in *X. laevis*.

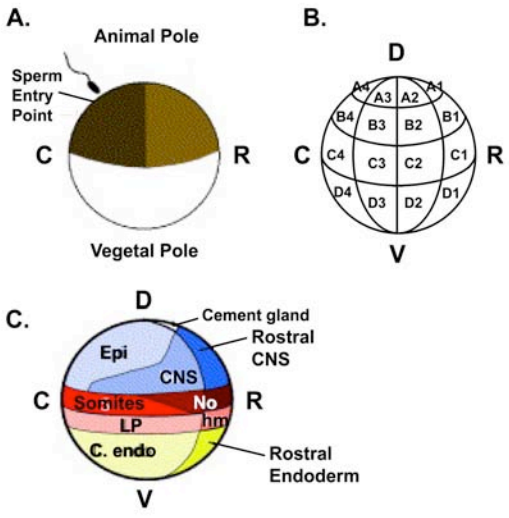


Figure A-1 Fate map of a 32-cell *Xenopus laevis* embryo. (A) Following fertilization and cortical rotation, cells derived from the lightly pigmented side of the embryo will form rostral (R) structures and the darkly pigmented side will give rise to more caudal (C) structures. This schematic represents a 90° rotation from the traditional dorsal/ventral (D/V) axis. It should be noted that neither the original fate map, nor the updated version is strictly accurate in assigning the D/V axis since dorsal and rostral fates are closely aligned, as are ventral and caudal fates. (B) Blastomere nomenclature of a 32-cell (stage 6) embryo (only cells in one of two sides with respect to the left-right axis are shown). (C) Prospective adult tissues derived from regions of a 32-cell embryo, as seen from a side view. C). Adapted with permission from (Lane and Sheets, 2006). D = Dorsal, V = Ventral, R = Rostral, C= Caudal, Epi = Epidermis, CNS = Central Nervous System, No = Notochord, LP = Lateral Plate Mesoderm, hm = head mesoderm, endo = endoderm.

In this chapter we describe methods to generate and culture *Xenopus* embryos, and to perform targeted injection of antisense morpholino oligonucleotides and capped mRNAs in order to manipulate gene expression in various tissues. It is worth noting that the microinjection techniques described herein are not limited to straightforward overexpression and knockdown of gene function, and are best applied in the context of established *Xenopus* resources for optimum utility. For example, many signaling pathways responsible for early vertebrate axis formation and tissue patterning (such as the bone morphogenetic protein (BMP) or the Wnt pathway) have been very well studied in *Xenopus*. The defects specific to alterations in these pathways may therefore be used as readouts for perturbations at particular steps of the given pathway. This phenomenon is well illustrated by studies that take advantage of the BMP signaling pathway. During *Xenopus* development, BMP is expressed in a caudal

to rostral gradient across the early embryo. High BMP expression on the caudal side of the embryo is required to specify ventral and caudal fates and misexpression of BMPs or molecules that are BMP downstream effectors on the rostral side of the early embryo causes characteristic ventralization of dorsal structures and caudalization of anterior structures (Dale et al., 1992; Jones et al., 1992). Conversely, injection of BMP inhibitors, or proteins that have a dominant negative effect on BMP signaling, on the caudal side of the embryo results in inappropriate dorsalization and rostralization in this region and produces a characteristic axis duplication (Maeno et al., 1994a; Suzuki et al., 1994). Overexpression or misexpression of genes in tissues where they are not normally expressed may thus provide clues to their normal function, depending on the effect that they have on an established molecular pathway or patterning event by changing downstream gene expression or morphology. Indeed, many genes of unknown function have been identified as naturally occurring agonists or antagonists of BMP function based on the dorsalized or ventralized phenotype that is observed when they are ectopically expressed.

A disadvantage of using injection of mRNAs is that there is poor control of transcriptional timing. Injected mRNAs are immediately translated and persist for many hours, and up to several days in some cases, in the embryo. Injection of cDNA expression constructs with tissue- or temporal-specific promoters is an alternative method for overexpression. cDNAs containing ubiquitously expressed viral promoters, such as the cytomegalovirus promoter, will not be transcribed until zygotic transcription initiates during the mid-blastula transition, so this

technique may be useful for determining the effect of later gene induction. However, this approach has the major drawback that expression of cDNA constructs is of variable efficiency and results in highly mosaic expression (Sargent and Mathers, 1991; Vize et al., 1991).

While different anti-sense technologies continue to be developed in a variety of systems, morpholinos have proven the most effective method for attaining reproducible knockdown of a specific gene (Heasman, 2002). Blocking a known signaling pathway may also be accomplished by injection of a dominant negative protein (as described above for BMP). It is important to note, however, that dominant-negative proteins (as is the case with a dominant negative BMP receptor) may affect other related proteins and family members and thus phenotypic effects may not be a result of inhibiting a single target.

Once gene expression has been altered by injection or by chemical exposure, tissues within individual treatment groups can be easily pooled, subject to microarray analysis (as discussed below) and analyzed for changes in global gene expression due to a specific perturbation. Although lack of a sequenced genome still poses a significant challenge when working with *Xenopus laevis*, the availability of genetic resources continues to be improved.

Considerations when performing a microarray analysis in *Xenopus laevis*

Xenopus laevis is an excellent system for controlling early gene expression (i.e. knockdown, upregulation and/or misregulation of specific genes) in a whole animal system and for gaining access to tissues at very early time points.

Because many embryos are obtained in a single spawning event, a large amount of material may be generated in a relatively short period of time. Experiments can thus be performed, and adequate quantities of sample for a microarray can be collected rapidly without relying on amplification techniques. However, because the genomic resources are scarce there tends to be very little or poor annotation for most genes on commercial *X. laevis* chips. It is therefore important to consider the annotation status for a particular chip before embarking on a resource-intensive study such as a microarray project. One may want to consider using a chip from the related organism *Xenopus tropicalis*, which shares a high degree homology with *X. laevis*. The *X. tropicalis* genome is not duplicated and it has thus been fully sequenced. Despite these drawbacks, a number of genetic resources have been developed lately (see (Ogino and Ochi, 2009) for a good review and comprehensive list) and progress continues to be made in understanding the *X. laevis* genome. Finally, because *Xenopus laevis* is not a clonal species, it is also important to recognize that there will be a high degree of background variability between individual frogs. It is therefore important to have enough biological replicates to identify significant targets above the background, and to have a good statistician on hand. In spite of these genomic limitations, its large degree of genetic conservation with higher organisms, short developmental timeline, and overall accessibility make it a very useful model system with which to efficiently study early events in vertebrate development.

2. Materials

Unless otherwise indicated, solutions should be prepared and stored at room temperature.

2.1 Generation and Testing of Morpholino Oligonucleotides

1. Morpholino oligonucleotides (GeneTools, LLC; see Methods for considerations regarding morpholino ordering and appropriate control morpholinos)

2.2 Generation of Synthetic mRNA for Microinjection

1. mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion). Polymerase-specific kits are available and selection depends on which RNA polymerase promoter is upstream of the gene of interest.
2. Equilibrated Phenol pH 8.0 (USB)
3. Sevag [Chloroform:Isoamylalcohol (24:1)]
4. 10M Ammonium Acetate
5. Ethanol
6. Isopropanol
7. Sephadex G-50 spin column (IBI Scientific). Optional but recommended
8. Linearized template cDNA

2.3 Collection of Testes

1. Tricaine (Sigma). 0.2% (w/v) dissolved in dH_2O (prepare fresh)

2. Modified Barth's Saline (MBS). For 10X stock: 880mM NaCl, 10mM KCl, 25mM $NaHCO_3$, 100mM HEPES (pH 7.5), 10mM $MgSO_4$, 0.14mM $Ca(NO_3)_2$, 0.41mM $CaCl_2$; Adjust to pH 7.5 with NaOH, filter sterilize and store at 4°C
3. Testis Buffer: 10% Fetal Bovine Serum, 1% Pen/Strep (100U/mL Penicillin, 100μg/mL Streptomycin; Sigma) in 1X MBS. Divide into 10mL aliquots and store at -20°C

2.4 Collection and Fertilization of Embryos

1. Powder-free, Latex-free Vinyl Gloves for handling frogs (*see* **Note 1**)
2. 1mL sterile syringe (Kendall)
3. 27 and 20 gauge sterile syringe needles (Kendall)
4. Human Chorionic Gonadotropin (Sigma). 4,000U/mL dissolved in sterile dH_2O; store at 4°C
5. Holtfreter's Frog Water. For 200X stock: 3M NaCl, 34mM KCl, 12.5mM $NaHCO_3$ 33.7mM $CaCl_2$. For 4L, dissolve NaCl, KCl, $NaHCO_3$ in 3L dH_2O. Dissolve 19.8g $CaCl_2 \cdot 2H_2O$ in 500mL dH_2O, add slowly to above and bring to 4L
6. Conical Tissue Grinder (Research Products International)
7. Aged Tap Water (Tap water, allowed to sit for at least 24 hours for chlorine to evaporate; optional as 0.1X MBS may be used instead)
8. Dejellying Solution (prepare fresh): 2% (w/v) Cysteine dissolved in dH_2O; Adjust to pH 7.8-8.0 with NaOH

9. DeBoer's Pond Water. For 20X stock: 100mM NaCl, 1.3mM KCl, 0.44mM $CaCl_2$; Adjust to pH 7.4 with $NaHCO_3$, and store at 4°C
10. Glass Petri dish (100 x 15mm)

2.5 Targeted Embryo Injection and Culture

1. Dissecting Microscope and transmitted light source with double gooseneck arms. A manually operated X-Y stage is optional but recommended, particularly if homemade injection trays (Item 7 below) are to be used (*see* Fig. A-2A, B)
2. Microinjector that can accurately deliver 10-100nL volumes. Narishige and Drummond make two good options. For this protocol, we describe injections using the Drummond Nanoject II.
3. Pulled glass capillaries (micropipettes; injection needles) used for sample injection; obtainable from companies that make microinjectors (Narishige L = 90mm, OD = 1mm, ID = 0.6mm; Drummond L = 3.5 inches, OD = 1.14mm, ID = 0.53mm)
4. Micropipette puller (Sutter)
5. 26 gauge Hamilton Syringe to be used for backfilling the micropipette needle with mineral oil (for use with Drummond Nanoject)
6. Low temperature (14-25°C) bioincubator is useful but not required
7. Homemade Injection Trays. These are optional but highly recommended to facilitate injection (*see* **Note 2** *and* Figure A-2C)

8. Wooden applicator sticks for manipulating and sorting embryos. Sticks can be shaped to a point using a boxcutter and then blunted slightly to avoid puncturing embryos during manipulation
9. Sterile Mineral Oil (Sigma; for use with Drummond Nanoject)
10. Ficoll solution. Ficoll (type 400 DL; Sigma) is dissolved to 5% w/v in 0.1X MBS; Adjust to pH 7.5
11. Gentamicin (50mg/mL; Gibco)

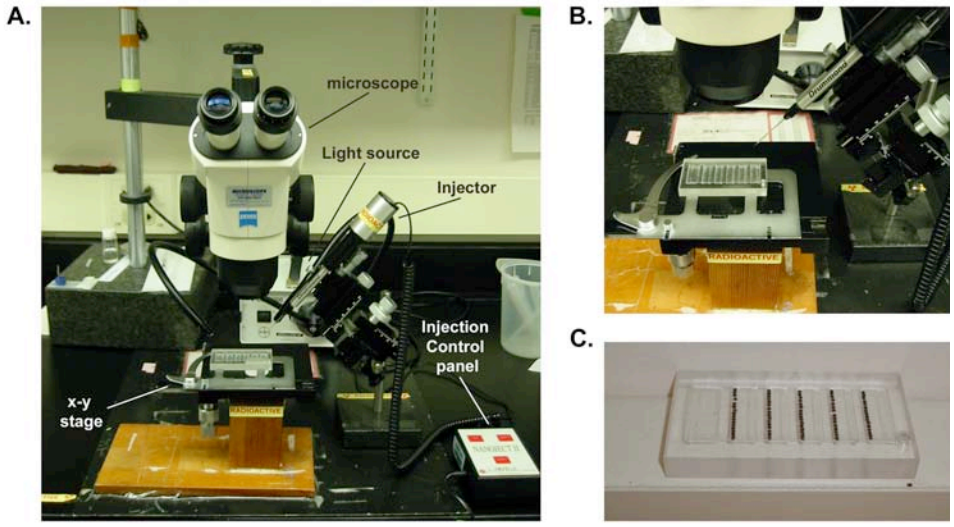


Figure A-2. Microinjection apparatus.
(A) Example of a microinjection set-up using a dissecting microscope, fiberoptic light source and an X-Y stage (B) Close-up view of the X-Y stage with injection tray and injection apparatus (Jones et al.). (C) Homemade injection tray with embryos prepared for injection in each of the five lanes.

3. Methods

3.1 Selection and Testing of Morpholino Oligonucleotides

Antisense morpholino oligonucleotides block gene function by either binding to sequence near the ATG start codon of an mRNA and inhibiting its translation, or by binding to the splice junction of a gene and preventing splicing. Because

complete sequence information, including splice junctions, is not available for *X. laevis*, translation-blocking morpholinos are more commonly used in this species. *X. laevis* is pseudotetraploid, having undergone a partial duplication of the genome and thus it is important to search available data bases [e.g. Xenbase (http://xenbase.org/genomes/blast.do?); Entrez (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene); The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi)] to determine whether there are multiple copies of your gene of interest and, if so, to identify sequences corresponding to both before designing MOs. MOs are purchased from GeneTools. The company provides instructions for optimal MO design on their website (http://www.gene-tools.com/node/18), or customers can upload relevant sequence information and GeneTools will identify the most appropriate target sequence. In most cases, it is necessary to either design one oligo that will block both copies of your gene of interest or, if this is not possible, to use a mixture of two oligos that will target both copies of the gene. A non-overlapping oligo may be used as a positive control, for additional verification of phenotype specificity. A standard negative control MO is available from GeneTools. Alternatively, mismatched MOs containing five mismatched bases distributed throughout the oligo sequence are a more stringent test of specificity. The appropriate dose is dependent upon the individual MO and must be determined empirically. To minimize potential toxic effects, we recommend starting at a low dose (1-5ng) and increasing as necessary. We have found that high doses of MO may be tolerated reasonably well (up to 100ng), depending on the particular MO.

1. MO solubility is an ongoing issue and appears to be dependent upon the individual oligonucleotide (*see* **Note 3**). MOs are shipped as lyophilized pellets. The MO is then resuspended, at a concentration appropriate to the particular use, in sterile (not DEPC-treated) dH_2O, and heated at 65°C for 10 minutes to bring it into solution. For *Xenopus* injections, we have found that 1mM stock dilutions are sufficiently concentrated for all required applications. Create appropriate working dilutions and store in individual aliquots at –80°C (*see* **Note 3**). Prior to use, heat aliquots at 65°C for 10 minutes and vortex briefly to ensure that all MO is in solution to obtain an accurate concentration. Spin tubes at top speed for 5 minutes in a microcentrifuge to remove insoluble material that may clog the injection needle.
2. There are several methods outlined below to verify that MOs specifically knockdown the intended target.
    i. Demonstrate reduction in protein synthesized by Immunohistochemistry (IHC) or Western blotting in morphants compared to control embryos.
    ii. In the absence of an adequate gene-specific antibody, demonstrate reduction of an overexpressed epitope-tagged protein in morphants compared to control embryos by IHC or Western blotting.

iii. If splice-blocking morpholinos are used, demonstrate that the targeted splicing event is inhibited via polymerase chain reaction (PCR) to detect loss of the spliced form in morphants.

iv. Ultimately the best test of specificity is to demonstrate that phenotype(s) attributed to knockdown of a particular gene are rescued by co-injection of a gene-specific mRNA that harbors silent mutations that prevent morpholino recognition.

3.2 Generation of Synthetic RNA for microinjection

mRNAs require a 5' 7-methylguanosine cap, and a 3' polyadenylate [poly(A)] tail to prevent degradation and promote efficient translation *in vivo*. For *in vitro* synthesized RNAs, the former is accomplished by addition of cap analog [m7G(5')ppp(5')G] to the synthesis reaction. A poly(A) tail can be added following the RNA synthesis reaction using a commercially available kit (e.g. Ambion poly(A) tailing kit) but is more commonly encoded in the cDNA or in the expression vector (e.g. pSP64T, obtainable from http://faculty.washington.edu/rtmoon/XE40.html (Zelus et al., 1989). Alternatively, polyadenylation can be accomplished *in vivo* by the inclusion of an SV40 polyadenylation signal in the expression vector. pCS2+ is a commonly used multipurpose expression vector that includes an SP6 promoter upstream, and an SV40 poly(A) signal downstream of the multiple cloning site, as well as a second polylinker with unique restriction sites for linearizing the template DNA downstream of the SV40 poly(A) signal (Rupp et

al., 1994; Turner and Weintraub, 1994). A number of pCS2+ derivatives have been constructed that allow fusions to epitope tags or reporter proteins such as ß-galactosidase to facilitate detection in downstream applications. As with MOs, the dose for a particular mRNA must also be empirically determined. RNA tends to be more toxic than MOs, thus much lower amounts (1-5pg) should be used initially when determining the appropriate dose.

1. Linearized DNA template should be prepared in advance and resuspended in H_2O or TE at 0.5μg/μL. An example protocol is as follows: Cut 20μg of template DNA in 100μL reaction volume with the appropriate restriction enzyme. Run 5μL on an agarose gel to verify that cutting is complete. Extract once with Phenol:Sevag (1:1) and once with Sevag alone. Add Ammonium Acetate to 0.4M and 2 volumes 100% EtOH. Precipitate overnight at -20°C, or for 15 minutes at -80°C. Spin 10 minutes at 4°C at top speed, rinse with 70% EtOH. Vacuum dry DNA pellet and resuspend in 36μL DEPC dH_2O. This will give a concentration of roughly 0.5μg/μL, assuming a 10% loss of input.
2. Capped mRNAs for overexpression and/or morpholino rescue are generated using Ambion's mMESSAGE mMACHINE High Yield Capped RNA transcription kit.
3. To prepare capped RNAs, add the following ingredients, in the order listed below, to a sterile 1.5mL tube at room temperature (if

tube is kept on ice, the spermidine in the buffer can precipitate the template DNA).

4µl DEPC treated dH_2O

10µl 2X rNTP Mix [includes m7G(5')ppp(5')G cap analog]

2µl 10X transcription buffer

2µl linearized template DNA (0.5µg/µl)

2µl T7 or T3 RNA polymerase for a total volume of 20µl.

4. Tap tube to mix and spin briefly to collect contents at the bottom of the tube.
5. Incubate at 37°C for two to four hours (*see* **Note 4**).
6. Add 1µl DNase (from kit), incubate at 37°C for 15 minutes.
7. Before use in injection, the capped mRNA must be purified from the reaction components, particularly from the cap analog, which will compete with transcription of full-length product (*see* **Note 5**). Bring up reaction volume to 50µL with 30µL DEPC dH_2O to minimize loss during the purification and do a single Phenol:Sevag (1:1) extraction.
8. During centrifugation for the Phenol:Sevag extraction, prepare sephadex G-50 spin columns according to manufacturer's instructions. Briefly, invert column to resuspend the gel. Remove caps on both ends of the column, place in a collection tube (provided) and spin at 1100 x g for 1 minute in a swinging bucket

centrifuge to drain excess buffer. Empty collection tube and repeat. Place column in new collection tube.

9. Following the Phenol:Sevag extraction, transfer the aqueous phase of the extraction (~50μL) to the center of the gel bed of the sephadex G-50 column and spin at 1100 x g for 4 minutes.
10. Transfer the G-50 column eluate to a new 1.5mL tube and precipitate the capped mRNA with 0.2M Ammonium acetate and two volumes of 100% EtOH at -80°C for 15 minutes. Spin at top speed in a microcentrifuge at 4°C for 10 minutes. Rinse pellet with ice-cold 70% EtOH, and vacuum dry briefly. Do not over dry.
11. Resuspend the small RNA pellet in 25μL DEPC dH_2O and determine mRNA concentration by UV spectroscopy. Run 300ng of capped mRNA on a 1X MOPS gel to determine that a single full-length transcript has been synthesized and to verify expected concentration (*see* **Note 6**).

3.3 Collection of Testes

1. To harvest testes for fertilization, a male is anesthetized in 0.2% Tricaine for 20-40 minutes until unresponsive when its claw is pinched firmly between your thumb and forefinger.
2. Use a pair of blunt forceps to pull the skin up from the body wall. A tissue placed over the prospective incision site to remove excess moisture may aid in grasping the slippery skin. Using either a surgical knife or a pair of fine-pointed scissors, make a horizontal

incision in the skin across the abdomen (*see* Fig. A-3A). Make a parallel incision in the underlying body wall, being careful not to damage the underlying organs. Make a third, this time vertical incision along the length of the abdomen, beginning at the midpoint of the horizontal incision and continuing anteriorly up the midline through both layers (*see* Fig. A-3A). Fold back the resulting flaps like the pages of a book (*see* Fig. A-3B). Clip ventricle of the heart with the scissors to exsanguinate and ensure death. To prevent contamination of the testes, take particular care to avoid perforating the gut during the dissection.

3. Using a blunt pair of forceps, move the overlying organs aside and pull the fat bodies out (Fig. A-3B, black arrow), following them back to either side to find the testes (Fig. A-3B, white arrow). Remove each testis by snipping it away from the fat bodies with scissors.
4. Once the testes are removed, carefully snip away any adherent fat bodies and vasculature and rinse in a petri dish filled with cold 1X MBS to remove remaining blood before storing in cold Testis Buffer.
5. For optimum results, use freshly isolated testes. Remaining testes may be stored at 4°C and used for up to two weeks, although sperm viability will decrease with time (*see* **Note 7**).
6. Dispose of the carcass appropriately according to IACUC regulations.

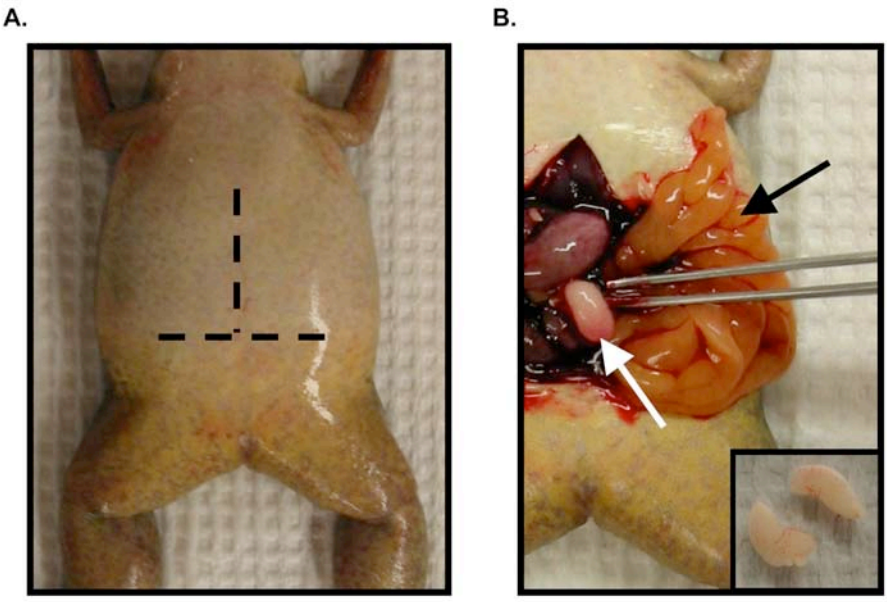


Figure A-3. Harvesting testes from a male frog.
(A) Ventral view of an anesthetized male frog. Dashed lines represent the approximate location of horizontal and vertical incisions to be made. (B) Testis removal following incisions in panel A and exsanguination (described in Methods). The testis (white arrow, and inset) is located at the end of the fat bodies (black arrow) on the left and right side of the abdominal cavity.

3.4 Collection and Fertilization of Embryos

1. To induce spawning, prime three mature females with 1200U human chorionic gonadotropin (HCG) solution by injecting 0.3mL of 400U/mL HCG per frog into the dorsal lymph sac (*see* Fig. A-4A and **Note 8**). Injected females are housed in plastic drawers or buckets in a 15°C bioincubator in 1X Holtfreter's Frog Bath overnight (*see* **Note 9**). Spawning will begin 12-14 hours later if they are kept at 15°C. It may be necessary to exchange the Frog Bath to bring the frogs up to room temperature more quickly if spawning does not begin. Alternatively, females can be injected the

day eggs will be used, housed at room temperature and will begin spawning within approximately six hours.

2. Collect eggs in a glass petri dish containing 1X MBS (*see* **Note 10**) To induce spawning, pick up the female in one hand, holding the rear legs anteriorly against her body with the index and middle fingers on either side of her torso (*see* Fig. A-4B). To keep the frog calm, cover the eyes and head with the palm of the same hand. *Gently* apply pressure to the abdomen with thumb. The other hand may be used to stabilize the animal on the ventral side and to apply gentle pressure laterally (*see* **Note 11**). More than one dish may be used if necessary to distinguish the eggs from individual frogs.
3. Just prior to fertilization, cut a small piece of testis (~1/5, from Section 3.3) and place it into a clean 1.5mL tube containing ~1mL 1X MBS. Crush with a conical tissue grinder to make a testis slurry, and store on ice until used up (~3 fertilizations). Alternatively, a small piece of whole testis may be used, as described below.
4. Remove as much of the 1X MBS as possible from the dish of eggs with a plastic transfer pipet, taking care to avoid touching the eggs, which may damage them as they are fragile at this stage. The jelly-like consistency of the egg mass will protect the eggs from excess surface tension until they are fully dejellied (step 5, this section). Add ~300μL testis slurry and ~1mL 0.1X MBS and agitate dish to ensure that solution reaches all eggs. The lower salt solution

facilitates fertilization by increasing sperm activity (*see* **Note 12**).Allow eggs and sperm to incubate together for 2-3 minutes in the ~1mL of 0.1X MBS to permit fertilization to take place. After fertilization, fill the dish with aged tap water or 0.1X MBS and let stand for about 20 minutes. During this time, the jelly coat will form such that the eggs adhere to each other and to the dish. Eggs that have been fertilized will rotate within the fertilization membrane such that the pigmented animal pole faces upward. From this time on, embryos may be kept at varying temperatures, ranging from 14-25°C, to control the rate of development as necessary (*see* **Note 13**). An approximation of the changes in developmental timing based on temperature are as follows: 25°C = 100%, 20°C ≈ 75%, 16°C ≈ 50%, and 14°C ≈ 25% normal developmental rate. Timing of normal development is based on staging by Nieuwkoop and Faber (Nieuwkoop et al., 1967).

5. To prevent the eggs from adhering to one another and to the needle during injection, it is important to fully dejelly the eggs. Once rotation is complete (~20 minutes) and prior to injection, embryos may be dejellied at any time. It should be noted, however, that empirical evidence suggests that cleavage patterns may be more regular if they are dejellied after they have cleaved at least once. Pour off the aged tap water or 0.1X MBS, and fill the dish with 2% cysteine solution (pH 7.8-8.0). Very gently agitate on an orbital

shaking platform for 3-5 minutes until the jelly coat is completely removed and the eggs are clustered tightly together in the center of the dish.

6. Once the eggs are dejellied, remove the cysteine solution using a plastic transfer pipet or by tilting the dish and pouring into a waste container. The eggs are free-floating and fragile at this point and thus it is important to tilt the dish in order to keep the eggs covered with a small volume of fluid at all times to reduce damage from surface tension. Using the same technique, rinse the eggs 2-3 times with 1X De Boer's solution and then 2 times with 0.1X MBS. Embryos can be cultured in 0.1X MBS throughout early development.

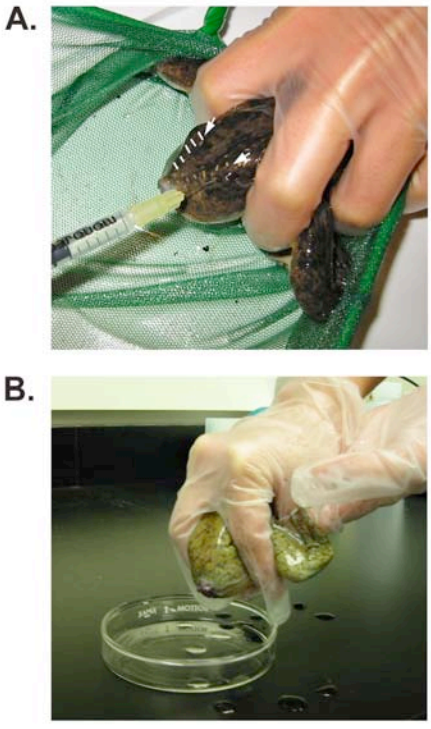


Figure A-4. Spawning eggs from a female frog.
(A) Priming a mature female by injection of human chorionic gonadotropin into the dorsal lymph sac on the posterior aspect of the frog. The injection needle is inserted just lateral to the V-shaped lateral line sutures (white arrows; dashed white lines demarcate the sutures on the left side of the frog's back). The head and eyes of the frog are covered with the palm of the hand (out of view) to reduce stress and the frog is immobilized by using a net to prevent forward motion and by holding the legs forward with two fingers. (B) Spawning of a female frog. The frog is held as described above, using the opposite hand, instead of the net, to provide additional stability.

3.5 Targeted Embryo Injection and Culture

There are many different small volume microinjectors available, but two commonly used apparati for injection of *Xenopus* eggs and oocytes are the Pico-injector (Medical Systems) and the Nanoject (Drummond Scientific). The Pico-injector uses compressed gas to reliably deliver nanoliter volumes through micropipettes by applying a regulated pressure for a digitally set period of time. For this method, the tip diameter of the micropipette is irrelevant, and thus finely tapered micropipettes with very small tip openings can be used to minimize damage to embryos, which is especially important when injecting single cells at late cleavage stages. A disadvantage is that injection volume must be calibrated for each new micropipette by measuring the size of the drop delivered using a stage micrometer. The Nanoject uses positive volume displacement to dispense preset volumes. This instrument is less costly, and has the advantage that one does not need to calculate drop size for each micropipette. Micropipettes must exceed a certain minimal tip diameter, however, and must also be back-filled with mineral oil prior to use. Below, we describe a protocol for use of the mineral oil-based volume displacement method. For a detailed description of the gas pressure-based injector see (Hazel L. Sive, 2000).

1. To generate needles for microinjection, glass capillaries are heated and pulled to a fine point using a needle puller. Specific settings will vary between needle pullers and some experimentation with the various parameters (i.e. heat, pull, velocity, etc.) is required.

However, once the appropriate settings for a given capillary have been established, many needles may be pulled quickly in case replacement is required during the injection day or for future experiments.

2. A good needle is sharp with a small bore at an angle of approximately 45°. Under a dissecting microscope, clip the tip of the needle using a pair of sharp forceps. The needle should be sharp but not so thin that the tip is flexible and will bend when it comes in contact with the embryo.
3. Backfill the needle with mineral oil using a Hamilton Syringe. Insert the syringe all the way into the tip of the needle. Applying constant pressure to the plunger, eject the oil while slowly withdrawing the syringe to avoid introducing bubbles, which will compromise the accuracy of the volume displacement method used by the injector (*see* **Note 14**).
4. Load the needle onto the injector taking care not to introduce bubbles. Be sure that the needle is loaded straight to avoid bending the recessed plunger on the injector, and apply gentle but firm pressure until the needle pops snugly into the rubber o-ring.
5. The injection control box has three buttons labeled “empty,” “fill,” and “inject.” The inject button may also be operated using a foot pedal, allowing for hands-free injection. Lay a piece of parafilm on the injection stage, clean side up. Depress the “empty” button to

eject one third to one half the volume of oil onto the parafilm in order to make room for the injection solution (*see* **Note 15**). Pipet a few microliters of the sample solution onto the parafilm and depress the fill button to draw sample into the needle, taking care to avoid air bubbles by keeping the needle submerged (*see* **Note 16**). When preparing to inject, depress the “inject” button to eject several droplets into air to determine if the injector is yielding droplets of roughly equivalent size.

6. Mount the injection tray on the injection stage (*see* Fig. A-2B) and fill with 5% Ficoll solution. Check that the injector is aligned properly with the stage such that the needle is positioned over the tray and tracks appropriately along each lane.
7. Using a disposable plastic transfer pipet, select and transfer embryos to the injection tray. When selecting embryos be sure to choose ones that look healthy (i.e. cleavage and pigmentation patterns are regular).
8. Arrange embryos of the desired stage in the injection tray, with the appropriate side facing the needle (e.g. if dorsal injection is desired, align the embryos with the dorsal side facing the injector).
9. The injection volume and speed are controlled by the orientation of a panel of dipsticks located on the right side of the injection control box. Find the key for dipstick settings and their corresponding

volumes and speeds on the bottom of the control box and set them appropriately.

10. Proceed with injection, periodically injecting into air to ensure that the needle is not clogged and droplets are of expected size.

11. Once injections are complete, culture the embryos in Ficoll solution for several hours to overnight to allow them to heal (*see* **Note 17**).

12. Rinse 2-3 times in 0.1X MBS and remove any unhealthy or dying embryos as they will compromise the health of the others in the dish, then culture embryos in 0.1X MBS. 50ug/mL gentamicin may be added in order to inhibit bacterial growth (optional).

13. Continue to culture embryos in 0.1X MBS (+/- gentamicin) until desired stage is reached. Culture solution should remain clear and be kept free of debris.

Notes

1. Frogs may be handled with clean hands or with vinyl gloves. Gloves made from other materials (e.g. latex) will damage the frogs' fragile skin. If gloves are not used, hands should be washed thoroughly before and after contact with the frogs to ensure that they are free from lotions and trace detergent, which can be damaging to the frog, and to remove bacteria that may have transferred from the skin of the frogs.
2. Though not required, we have found it extremely useful to use homemade injection trays (Fig. A-2C) to hold embryos and oocytes for injection. These trays can be ordered from any custom acrylic fabricator and will be manufactured to specification. Our trays consist of a 7.5 x 4.0 x 1.0cm acrylic block that is hollowed out to a depth of 4mm. Narrow strips of acrylic (4mm wide and either 1 or 2mm high) are placed 2mm apart in an alternating fashion to generate a trough in which one side is taller than the other; this allows the embryo to rest against the taller side while being injected on the opposite side (Fig. A-2B, C). Embryos can be lined up in recessed lanes, allowing them to be completely submerged in Ficoll solution without floating about. This ensures that they are not subject to excess surface tension, yet are still held in place so that keeping track of which embryos have been injected is not an issue. A number of other techniques have also been used to hold embryos in place during injection. In the most basic setting, embryos can be placed in a petri dish and, using a pair of blunt forceps, positioned and stabilized appropriately for injection.

Following injection, the embryos are placed to one side of the dish to keep track of which ones have been injected. Alternatively, a plastic petri dish may be fitted with a nylon mesh and used to hold embryos in place (Hazel L. Sive, 2000).

3. GeneTools recommends storing MO stocks and working dilutions at room temperature and, if necessary, heating them at 65°C for 10 minutes prior to use (http://www.gene-tools.com/files/Essential%20INFO%2002-09.pdf). We have found that while some oligos are stable (i.e. they generate reproducible results) when stored at room temperature, we have also found that both the more concentrated stocks and working dilutions of certain oligos have given more reproducible results when stored at –80°C and heated at 65°C for 10 minutes prior to use.
4. It is necessary to determine the appropriate cap analog:GTP ratio for your particular transcript. Increasing cap analog increases the fraction of capped transcripts but reduces yield. Yield is also dependent on the efficiency of the particular transcription enzyme (i.e. SP6, T7 or T3). We bias the reaction toward increasing the amount of capped transcript, as uncapped mRNA is rapidly degraded in the embryo. Ambion provides a very detailed protocol entitled “Synthesis of Capped RNA Transcripts” in the mMESSAGE mMACHINE manual, which describes the potential benefits to varying cap analog:GTP ratio and incubation time, which may be useful.

5. As an alternative to purification of capped mRNA on a sephadex column, nucleotide removal may also be accomplished by isopropanol precipitation after DNase treatment as follows: Add 115µl RNase free water and 15µl Ammonium acetate stop solution (from kit). In a fume hood, add 75µl Tris-buffered phenol and 75µl Sevag. Vortex well to mix. Microcentrifuge at 4°C for 5 minutes. Remove the aqueous top layer to a new 1.5mL tube. Discard the bottom organic layer into an appropriate organic waste container. Add 150µl 100% isopropanol, mix by inverting tube and allow RNA to precipitate at -20°C for 15 minutes to 1 hour. Spin at 4°C in a microcentrifuge for 15 minutes at top speed. Remove and discard the supernatant. Rinse pellet with 70% ethanol, spin briefly, remove all supernatant and resuspend in 25µL DEPC-treated dH_2O.
6. The expected yield for a single reaction starting with 0.5µg cDNA template is 15-20µg capped mRNA. The Ambion manual provided with the mMESSAGE mMACHINE kit provides several useful sections on troubleshooting various issues with capped RNA synthesis. Low RNA yield may be due to several factors. Increasing the incubation time to 4-6 hours, the amount of template, and/or the amount of polymerase are simple first steps to try. We have found that the quality and purity of the cDNA template is very important. Preparing new plasmid DNA or repurifying the cut template by phenol/chloroform extraction and ethanol precipitation often resolves yield issues for established transcripts. For transcripts that are being used for the first time, it may be necessary to

empirically determine the appropriate ratio of cap analog:GTP as addition of the cap analog dramatically reduces yield. Some reactions may yield multiple products. Multiple bands on the gel may be due to persistent mRNA secondary structure from incomplete denaturation on the gel or to an artifact of electrophoresis, or to premature termination of the mRNA synthesis reaction. In the case of the latter, the incomplete product will lack a polyA tail and will likely be degraded once injected. It is, however, important to adjust the concentration for injection appropriately so that it reflects only the full-length product.

7. To check sperm viability, the testis may homogenized in 1X MBS and a small amount of sperm may be diluted in 0.1X MBS, transferred to a glass slide and examined for motility under a light microscope using a 10X objective.
8. We prime three females per experiment day to ensure that enough eggs are produced for a particular day's experiments. We recommend priming at least two frogs, as there is significant variability between individual frogs as to how well they spawn and the quality of eggs laid. When performing an experiment (such as a microarray analysis) in which minimizing background variability is essential, we recommend using the eggs from a single female.
9. If older females are used, ovary development may be stimulated by pre-priming frogs 3-10 days prior to desired injection day with 50U Pregnant Mare Serum Gonadotropin (PMSG; Sigma) injected into the dorsal lymph

sac. Females will need to be temporarily kept in a separate holding tank to distinguish them from females that have not been pre-primed.

10. In the wild, eggs are spawned into fresh water, which induces formation of a jelly coat around the embryos. The jelly coat serves several functions, including providing mechanical support, acting as a block to polyspermy and activating the acrosomal reaction required for fertilization. To facilitate *in vitro* fertilization, eggs are spawned into a high salt solution (1X MBS) to prevent formation of the jelly coat. Eggs may also be spawned into a dry petri dish but care must be taken to avoid splashing water into the dish as this will induce instantaneous formation of a jelly coat and inhibit subsequent fertilization.
11. The cloaca of a female that is spawning will be dark red or pink in color. Females that are not spawning should not be squeezed. To induce spawning it may necessary to gently squeeze the frog as described in the methods. However, it is absolutely necessary to be gentle when squeezing and one must be sure not to press on the back of the animal as this may cause injury. Alternatively, the frog may push out the eggs when you pick her up. In this case, it is only necessary to stabilize her, using the same technique described above, while holding her over the egg collection dish.
12. Alternatively, testis may be used directly to fertilize eggs individually. Remove 1X MBS, add ~1mL 0.1X and use a small piece of testis to touch each of the eggs. Proceed as described.

13. Embryos may be allowed to develop at a range of temperatures, from 14-23°C. This allows for control of the rate of development to facilitate injections at a particular stage, or to produce embryos at varying stages simultaneously. While changing the incubation temperature does not cause problems for most applications, it is important to include the appropriate controls to ensure that your particular experimental output is not sensitive to changes in temperature.
14. Introduction of air bubbles into the injection needle is a common issue with this apparatus. Some tips to avoid introducing bubbles are as follows: 1) When backfilling the needle with mineral oil, be sure that the tip of the syringe is inserted as far as possible into the tip of the needle. Begin ejecting oil firmly *before* beginning to withdraw the syringe and continue ejecting oil until the syringe is completely withdrawn. Excess oil spilling out of the back of the needle can be blotted off with a tissue later. 2) When loading the needle onto the injector use a single upward movement. Avoid moving the needle up and down onto the plunger as this will also introduce unwanted bubbles. When seating the needle in the o-rings, take care not to chip the back of the needle as this will compromise the seal between the needle and the rubber o-ring.
15. A very small tip on the needle may prevent the mineral oil from being ejected effectively, if at all. If this is the case, carefully clip the needle back with sharp forceps to a 45° angle (this can be done on the injector if you're

careful) and attempt to eject the mineral oil. Repeat until the mineral oil is able to be ejected consistently.

16. When filling the needle with sample, keep the tip submerged in the sample and fill slowly. The plunger withdraws more quickly than either the solution or the mineral oil can pass through the needle, particularly when the tip is very small. This creates a vacuum, which can result in bubbles if the sample is depleted too quickly during filling. Pausing occasionally to allow the mineral oil and sample to catch up prevents this issue. A very small bubble or two may be unavoidable. If this is the case, be sure that the size of the bubble is minimized by depressing the eject button until sample starts to exit the tip of the needle. This will ensure that compression of the gas in the bubble does not affect the volume of oil displaced by the plunger during injection. A few small bubbles may not pose a significant problem, but if they become too numerous or too large, the needle will have to be reloaded.
17. Prolonged culture in Ficoll, while not detrimental, may affect the rate of development. Therefore, it may be necessary to also incubate controls in Ficoll. We recommend prompt removal from Ficoll solution once embryos have healed for several hours.

Acknowledgements

This work was supported by grants from the NIH (RO3HD058841 and RO1HD37976) and Shriners Hospital Research Foundation to JLC. MM was supported by NIH grant T32HD049309.

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